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THE RELATIONSHIP BETWEEN THE SEROLOGICAL REACTIONS AND THE INFECTIVITY OF POTATO VIRUS "X".

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QUANTITATIVE RELATIONSHIP BETWEEN VIRUS AND ANTIGEN.

BEALE (1934), working with the virus of tobacco mosaic, has found that a quantitative relationship exists between the antigen content of virus suspensions and the concentration of active virus contained in them as measured by local lesion counts. The virus suspensions used were filtrates of expressed sap, and the antigen content was measured by determining the precipitation end-point. In the experiments to be described below, dealing with the potato virus "X" (Smith, 1931), a different technique was adopted, but results in agreement with those of Beale were obtained. The titrations of antigen were made by the optimal proportion method, and the virus suspensions used were freed from the major portion of plant protein by the CO_2 precipitation method described by MacClement (1934) for the purification of virus "X". It has been found that crude filtrates of expressed sap are liable to precipitate spontaneously, more especially in the water-bath, and by using such purified suspensions it is believed that possible inaccuracies due to this precipitation and to any reactions between normal plant proteins and the anti-sera have been greatly reduced.

Spooner and Bawden (1935) showed that antisera have been obtained which react specifically with suspensions of potato virus "X", and that in constant antigen flocculation experiments with such sera zones of optimal flocculation could be obtained. The S strain of potato "X" (Salaman, 1933) produces necrotic local lesions on *Nicotiana glutinosa*, which, although neither so well defined nor so numerous as those of tobacco mosaic, can be counted with a reasonable degree of accuracy. A series of experiments has therefore been carried out with purified suspensions of the S strain, to determine the relationship between their antigen contents as indicated by optimal flocculation points and their virus contents as measured by local lesion counts.

The number of local lesions produced by one given suspension of the virus has been found to vary greatly with individual *N. glutinosa* plants, and also,

but within narrower limits, with the position of the inoculated leaf on the stem. In order to reduce the error caused by this variation the leaf and not the plant was treated as a unit in all local lesion experiments, and the virus suspensions to be compared were systematically varied on the leaves in a manner similar to that recently proposed by Youden and Beale (1934). The inoculations in each experiment were arranged so that each suspension was applied an equal number of times to leaves on each plant, and an equal number of times to leaves occupying the same relative positions on the stems of different plants. The following diagram, in which the vertical columns represent the leaves on a single plant and the horizontal columns leaves occupying the same relative position on the stem, shows the arrangement of the inoculations in an experiment in which twelve plants were inoculated with four virus suspensions, A, B, C and D :

A	B	C	D	A	B	C	D	A	B	C	D
B	C	D	A	B	C	D	A	B	C	D	A
C	D	A	B	C	D	A	B	C	D	A	B
D	A	B	C	D	A	B	C	D	A	B	C

The plants used in each experiment were all of the same age, and of approximately the same size. The growing points and all leaves not to be used were removed before the inoculations were made, and the leaves were washed in a stream of water from a wash-bottle immediately after inoculation. The virus suspensions were applied with glass spatulæ similar to those described by Samuel and Bald (1933), and were rubbed as evenly as possible over the whole leaf surface.

The flocculation experiments were carried out in 7 mm. thick-walled glass tubes, 0·9 c.c. of antigen at constant dilution being added to a series of tubes each containing 0·9 c.c. of antiserum at various dilutions ranging from 1/4 to 1/512. The tubes were immediately placed in a water-bath at 50° C. in such a manner that convection currents kept the mixtures continuously agitated. The bath was illuminated from behind so that the tubes could be continuously observed without being moved. The optimal tube was taken as that which first showed any visible flocculation. This could also be identified for some time after flocculation was apparent in other tubes as that containing the largest floccules, but towards the end of an experiment the optimum usually became obscured.

Table I shows the summarized results obtained from fifteen experiments in which purified suspensions of the S strain of potato virus " X " were compared for their flocculating optima with the same antisera, and for their capacity to produce local lesions. The virus used in all the experiments was derived from one original infected plant. A great number of infections has been made with virus derived from this source, and as the results obtained have shown extremely little variation, it is believed to be a relatively pure stock of the S strain. The virus suspensions compared in any one experiment, however, were not dilutions of one suspension. On the contrary, they were prepared at different times, from different host plants, and the virus after precipitation with CO_2 was resuspended in different amounts of water.

All the dilutions of antigen, antisera and inocula were made with 0·85 p.c. saline. In each experiment all the antigens were diluted similarly, viz. either 1/8 or 1/32, and they were diluted a further 1/4 before inoculation to *N. glutinosa*. Although the number of local lesions obtained by inoculating such diluted suspensions is relatively small, it is believed that more accurate comparisons of virus content have been obtained by this method of working, for it has been found that the number of lesions obtained with dilute suspensions varies much more directly with virus dilution than it does with more concentrated suspensions. In order to bring the lesion-counts to a more comparable basis, the number of lesions produced by each virus suspension is expressed in the table as the percentage of the total number of lesions obtained in that particular experiment.

From these results it will be seen that the more infective suspensions were those containing the more antigen, as indicated by the greater concentration of antiserum at the optimal flocculation point. In the majority of the experiments the strengths of the suspensions compared are found to lie in the same order when measured by both methods, whilst in some tests the relation between the dilution of antiserum in the optimal tube and the number of local lesions produced is almost a linear one. It would seem that in the virus suspensions examined a close relationship exists between the concentration of virus and antigen, and that the antisera provide a rapid and convenient method of determining the relative concentrations of such suspensions.

This close relationship, however, has been found to exist only when suspensions prepared in the same manner and presumably similar in all respects other than their virus content were compared. The results given in Table I were all obtained with suspensions purified by CO_2 precipitation, but when such pure suspensions were compared with Kieselguhr filtrates of crude infected sap, the two methods of estimating concentration frequently gave different results. It is possible that the differing hydrogen ion, protein and salt concentrations of the two types of suspension affect the number of lesions produced by a given amount of virus, and also the presence of comparatively large amounts of host plant protein in the Kieselguhr filtrates may affect the flocculation optima.

In order to obtain agreement between the two methods of estimating concentration, it has also been found necessary to work with stocks of the "X" virus which produce similar symptoms, and which contain the same strain or mixture of strains. Salaman (1933) has isolated three strains of virus "X", viz. S, L and G, of which only S produces necrotic local lesions on *N. glutinosa*. The three, however, cannot be distinguished from one another serologically, and the G strain, which produces only a faint systemic mottling in *N. glutinosa*, is antigenically equal to the S strain (Spooner and Bawden, 1935). When working with suspensions of virus "X" in which the strains were mixed in different proportions, it was found possible to obtain the same optimal flocculation points with suspensions giving widely different numbers of local lesions.

Table II shows the results obtained from such an experiment, in which suspensions of the S and G strains, and mixtures containing varying amounts of the two, were compared for their flocculating optima with an anti-S serum,

TABLE I.—*The Relation Between the Flocculating Optima and Infectivity of CO_2 Pure Suspensions of the S Strain of Potato Virus "X".*

Experiment No. and serum.	Number of virus suspensions.	Number of leaves inoculated with each.	Total number of lesions.	Suspension.	Serum dilution in optimal tube.	Percentage of total lesions produced by each suspension.
1	3	9	207	A	1/16	56
Anti-S				B	1/32	32
serum				C	1/64	12
2027						
2	4	12	1663	A	1/8	43
Anti-S				B	1/8	39
serum				C	1/64	10
2028				D	1/64	8
3	3	6	391	A	1/16	64
Anti-S				B	1/32	29
serum				C	1/128	7
2028						
4	6	12	474	A	1/8	40
Anti-S				B	1/16	25
serum				C	1/64	12
2028				D	1/64	11
				E	1/64	10
				F	1/256	2
5	2	12	70	A	1/64	80
Anti-G				B	1/128	20
serum						
2213						
6	3	9	78	A	1/32	50
Anti-S				B	1/64	29
serum				C	1/128	21
2030						
7	4	8	221	A	1/32	40
Anti-G				B	1/32	36
serum				C	1/128	13
2211				D	1/128	11
8	2	10	56	A	1/32	59
Anti-S				B	1/128	41
serum						
2030						
9	3	9	378	A	1/8	57
Anti-G				B	1/16	38
serum				C	1/64	5
2211						
10	4	8	70	A	1/8	66
Anti-G				B	1/16	28
serum				C	1/64	6
2211				D	1/256	0
11	4	8	363	A	1/16	53
Anti-S				B	1/32	29
serum				C	1/64	11
2030				D	1/128	7
12	4	8	360	A	1/32	43
Anti-G				B	1/32	42
serum				C	1/64	2
2211				D	1/128	13
13	4	8	254	A	1/32	47
Anti-S				B	1/64	31
serum				C	1/128	14
2027				D	1/256	8
14	4	8	624	A	1/32	53*
Anti-G				B	1/32	22
serum				C	1/32	18
2211				D	1/128	7
15	4	8	310	A	1/32	33
Anti-G				B	1/32	28
serum				C	1/32	26
2213				D	1/64	13

* Seven leaves inoculated with this suspension gave a total of 164 lesions, whilst the eighth leaf alone gave 169 lesions; the reason for this variation is unknown.

and for their capacities to produce local lesions. The antigens in each test were used at a dilution of 1/8 with saline and the inocula at 1/32, and the inoculations of the different mixtures were systematically varied on the leaves of ten *N. glutinosa* plants.

TABLE II.—*Comparison of S and G Strains, and Different Mixtures of the Two, for Flocculating Optima and Production of Local Lesions.*

Antigen.	Time.	Serum dilution. 1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	0.	Number of lesions.
G	6 min.	−	−	+	−	−	−	−	−	0
	11 „	+	++	+++	++	+	+	−	−	
	20 „	+++	++++	++++	++++	+++	+++	++	−	
3/4 G	5 „	−	−	+	−	−	−	−	−	13
1/4 S	9 „	+	++	+++	++	+	+	−	−	
	20 „	+++	++++	++++	++++	+++	+++	+	−	
1/2 G	7 „	−	−	+	−	−	−	−	−	20
1/2 S	11 „	−	+	++	+	+	−	−	−	
	20 „	++	++++	++++	++++	+++	++	+	−	
1/4 G	6 „	−	−	+	−	−	−	−	−	63
3/4 S	10 „	−	+	++	+	−	−	−	−	
	18 „	++	++++	++++	++++	+++	++	+	−	
S	8 „	−	−	+	−	−	−	−	−	120
	12 „	−	+	++	+	+	−	−	−	
	20 „	++	+++	++++	++++	+++	+++	+	−	

+ Signs indicate the degree of flocculation.

Each of the mixtures tested contained a similar amount of virus suspension, and this is indicated by the constancy of the optimal flocculation points (serum dilution 1/32), but not by the number of lesions. From these results it seems that with the serological method the total amount of antigen in such mixtures is measured, whilst the lesion count method estimates only that fraction capable of producing local lesions. They also indicate that the serological technique may be applied to viruses such as the G strain of potato virus "X" which have not this capacity, and with such viruses it should prove particularly useful in providing a convenient method of quantitative working.

Recent work has shown that many viruses are represented by a variety of different strains, which may exist together naturally in single infected plants. From the above results it is obvious that if such strains differ in their capacities to produce local lesions, as do those of potato virus "X", the lesion count method of comparing virus concentrations is liable to give extremely misleading results as to the relative virus content of different mixtures. Strains of the same plant virus, however, seem to be serologically similar (Spooner and Bawden, 1935; Chester, 1935), and the antisera should therefore also give a reliable quantitative method of working with virus cultures which are mixtures of such strains.

In an attempt to investigate further the relationship between the virus and the antigen a series of experiments has been made to determine their respective reactions to certain in-vitro tests.

EFFECT OF HEAT ON FLOCCULATION AND INFECTIVITY.

1 c.c. samples of purified S antigen were heated for 10 minutes in thin-walled glass tubes in a water-bath kept constant at various temperatures. They were then rapidly cooled, diluted 1/8 with saline and immediately used in constant antigen flocculation tests. A portion of the remaining antigen was further diluted 1/4 and inoculated to *N. glutinosa* plants for local lesion counts, whilst the remainder was inoculated without further dilution to six tobacco plants.

TABLE III.—*Effect of Heat.*

Treatment.	Serum dilution in optimal tube.	Number of local lesions.	Infectivity: Number infected/number inoculated.
Unheated	1/16	248	6/6
10 min. at 62° C.	1/32	117	6/6
10 min. at 64° C.	1/128	26	6/6
10 min. at 66° C.	No flocculation	0	0/6
10 min. at 68° C.	,,	0	0/6

Table III shows the results from an experiment in which heated purified S suspensions were titrated against an anti-G serum and inoculated to 10 plants, the inoculations being systematically varied as described above. Six other experiments with the S strain have given similar results. Heating above 66° C. always removed both infectivity and flocculating power, whilst heating between 60° C. and 66° C. shifted the optimum towards the region of more dilute serum, and reduced infectivity as measured by production of local lesions.

In two experiments with the G strain of virus "X" heating between 60° C. and 66° C. similarly shifted the optimum point, and heating above 66° C. destroyed infectivity and prevented flocculation.

The effect of ageing *in vitro* is apparently similar to that of heating. Two purified suspensions which were highly infective and flocculated actively with antiserum when freshly prepared were still infective after six months' storage, but flocculated less actively, whilst after fourteen months they were found to be non-infective and gave no flocculation with antiserum.

EFFECT OF FORMALDEHYDE ON FLOCCULATION AND INFECTIVITY.

The source of formaldehyde was commercial formalin, in which the amount of formaldehyde was found by iodine titration to be 35·8 p.c. 1 c.c. samples of purified S strain virus were mixed with 1 c.c. of formaldehyde at various concentrations to give a series in which the final dilutions of formaldehyde ranged from 0·139 p.c. to 8·9 p.c. The mixtures were placed at 1° C. for 48 hours, when they were diluted 1/4 with saline, used in constant antigen dilution experiments and also inoculated to six tobacco plants. The leaves were washed immediately after inoculation to prevent damage by the formaldehyde.

TABLE IV.—*Effect of Formaldehyde.*

Treatment.	Time.	Serum dilution. 1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	0.	Infectivity.
Control; no formaldehyde	15 min.	−	−	+	−	−	−	−	−	6/6
	20 „	+	+	++	+	+	−	−	−	
	45 „	+++	++++	++++	++++	+++	++	++	−	
0·139 p.c. formaldehyde	10 „	−	−	+	−	−	−	−	−	2/6
	15 „	−	+	++	+	+	−	−	−	
	40 „	+++	++++	++++	++++	+++	+++	++	−	
0·559 p.c. formaldehyde	15 „	−	−	+	−	−	−	−	−	0/6
	22 „	+	++	+++	++	+	−	−	−	
	45 „	+++	++++	++++	++++	+++	+++	+	−	
1 p.c. formaldehyde	15 „	−	−	+	−	−	−	−	−	0/6
	23 „	−	+	++	+	+	−	−	−	
	50 „	++++	++++	++++	++++	+++	++	+	−	
2·23 p.c. formaldehyde	20 „	−	−	+	−	−	−	−	−	0/6
	50 „	+++	++++	++++	++++	+++	++	+	−	
8·0 p.c. formaldehyde	20 „	−	−	+	+	−	−	−	−	0/6
	50 „	++	+++	+++	+++	+++	++	++	−	

Five further experiments were made with purified S suspensions and one with G, and similar results obtained, in each the flocculation with anti-viral sera being unaffected by the loss of infectivity. One sample of the S strain of virus "X" in 1 p.c. formaldehyde was not infective an hour after mixing, but its flocculation with immune sera was only slightly reduced after 16 weeks.

Gratia (1934) states that the flocculation of tobacco mosaic antigen with its antiserum is similarly unaffected by the presence of formalin, but gives no data as to the infectivity of the mixtures, whilst Matsumoto and Somazawa (1931) found that after infectivity was destroyed with formalin the suspensions still reacted with antiserum, but less actively than the controls.

EFFECT OF PHENOL ON FLOCCULATION AND INFECTIVITY.

Mixtures of phenol and purified S suspensions were made as follows to give suspensions of the virus in 2 p.c., 3 p.c. and 4 p.c. phenol:

1 c.c. virus + 2 c.c. 5 p.c. phenol + 2 c.c. saline.
1 c.c. „ + 3 c.c. „ + 1 c.c. „
1 c.c. „ + 4 c.c. „

The mixtures were placed at 1° C. for 48 hours, when 3 c.c. of saline were added to each and they were used in constant antigen flocculation tests, and also inoculated to six tobacco plants, the leaves of which were washed immediately after inoculation.

In four other experiments with similar virus suspensions it was also found that infectivity and flocculation with antisera were unaffected by 2 p.c. phenol, whilst in 3 p.c. and 4 p.c. the virus was not infective, and flocculation was greatly delayed and occurred only in the tubes at or near the optimal point. The amount of precipitate in these tubes was small and finely granular

TABLE V.—*Effect of Phenol.*

Antigen.	Time.	Serum dilution. 1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	0.	Infectivity.
S	8 min.	−	+	−	−	−	−	−	6/6
2 p.c. phenol	15 „	+	++	+	+	−	−	−	
	25 „	++	++++	+++	+++	++	+	−	
S	2 hours	−	+	−	−	−	−	−	0/6
3 p.c. phenol	24 „	+	+	+	−	−	−	−	
S	2 „	−	+	−	−	−	−	−	0/6
4 p.c. phenol	24 „	+	+	+	−	−	−	−	

instead of the typical large loose floccules. In two further experiments with weaker antigens normal flocculation was also obtained in 2 p.c. phenol, but in 3 p.c. and 4 p.c. no reaction with antisera was obtained even when the tubes were left in the water-bath for 24 hours. It seems therefore that when the virus is inactivated by phenol the antigen (or haptene) is not completely destroyed, but the system is in some manner affected so that flocculation is obtained only in concentrated mixtures and under optimum conditions.

EFFECT OF ALCOHOL ON FLOCCULATION AND INFECTIVITY.

On the addition of ethyl alcohol to purified suspensions of virus "X" a filmy gelatinous precipitate is produced. This is readily thrown down by centrifuging, and immediately goes into suspension on the addition of saline. In 50 p.c. alcohol it was found that the virus was precipitated completely: the supernatant after centrifuging was non-infective and also failed to react with antiviral sera, whilst the resuspended precipitate was highly infective and gave the same flocculation optimum as the original suspension.

In testing the effect of alcohol on the virus, the mixtures of virus and alcohol at various concentrations were placed at 1° C. for 48 hours, after which they were lightly centrifuged and the supernatants discarded. The precipitates after resuspension in saline were used in constant antigen flocculation tests, and also inoculated to tobacco plants. By this method it was found that in their reaction to alcohol the infectivity and serological reactions of potato virus "X" are closely related, both being destroyed by 85 p.c. ethyl alcohol.

Precipitates are also formed in the purified virus suspensions when they are dialysed and also when half saturated with ammonium sulphate. With these methods of precipitation, as with alcohol, it was found that the virus and the antigen behaved similarly. After centrifuging, the supernatant fluids were not infective and did not react with antiviral sera, whilst the precipitates when resuspended in saline were highly infective, and reacted with sera in a manner similar to that of the original suspensions.

SUMMARY.

A close relationship exists between the antigen content of purified suspensions of the S strain of potato virus "X", as measured by their optimal flocculation points with antisera, and their virus content as measured by the local lesion method. The strengths of such suspensions were found to lie in

the same order when compared by both methods. The relationship was found to hold only when the suspensions compared were prepared in the same manner.

The two methods of estimating concentration gave different results when different mixtures of strains of virus "X" were compared. Strains G and L are serologically indistinguishable from strain S, but produce no local lesions. It is suggested that the antisera provide a quantitative method of working with such viruses, and that they also afford a reliable estimate of the total virus in a mixture of strains, where the results from lesion counts would be misleading.

Inactivation of the virus by heat, ageing and alcohol was accompanied by the loss of flocculating power with antiviral sera. Inactivation by formalin left the flocculating power unimpaired, whilst inactivation by phenol greatly reduced and altered the flocculation, but did not completely destroy it. The antigen and the virus behaved similarly in their reaction to certain precipitants.

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EXPERIMENTAL ANÆMIA IN MONKEYS, WITH SPECIAL REFERENCE TO MACROCYTIC NUTRITIONAL ANÆMIA.

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THE following experiments were planned to determine the suitability of the *rhesus* monkey for studies in anæmia and to produce in them, by dietetic means, a macrocytic anæmia of the same nature as tropical macrocytic anæmia in man, in order to have an experimental animal for work on the nature of the curative factor in marmite. Previous experiments in which a macrocytic anæmia had been produced in these animals by faulty feeding (Wills and Bilimoria, 1932) were criticized on the ground that it had not been demonstrated that the *rhesus* monkey responded in the same way as man to other anæmia-producing stimuli. Such a demonstration, it was argued, was necessary on account of the very different responses called forth by such stimuli in the smaller laboratory animals. For example, chronic hæmorrhage, which in man produces a hypochromic microcytic anæmia, in rabbits may produce a macrocytic or normocytic anæmia (Steele, 1933; Price-Jones, 1911). A macrocytic anæmia in such animals cannot therefore have the same significance as in man, and until it has been shown that the hæmopoietic system of a given animal resembles that of man in the nature of its responses, it is unjustifiable to compare an experimentally-induced anæmia of the animal with a human disease.

In man there are two very distinct morphological types of anæmia, the macrocytic hyperchromic, *e. g.* pernicious anæmia, and the microcytic hypochromic, *e. g.* anæmia secondary to chronic blood loss. The former is associated with a megaloblastic reaction in the bone-marrow, the latter with a normoblastic reaction. In addition to these two types there are two further groups of anæmia; the normocytic, associated either with a more or less normal bone-marrow or with an aplasia, and the simple microcytic which shows no characteristic changes. These two groups are, with the exception of the aplastic type, also more indefinite in their blood picture; so for the purposes of this paper only anæmias belonging to the two former groups will be described in detail.

Methods.

The experimental animals were immature *rhesus* monkeys, at or just after the stage of the second dentition. They were kept in separate cages and were fed on the stock diet for some weeks before the experimental period. This was necessary in order to separate any animals suffering from obvious

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infections from the healthy stock, but even this precaution did not prevent one or two developing tuberculosis after they had started the experimental diet. The animals were weighed weekly. Blood was taken from the ear, so all the figures quoted are for capillary blood. It was found that the blood must be flowing freely from the ear or unreliable results were obtained. The same standardized pipettes were used throughout the work. Hayem's solution diluted 3 to 2 with water was found satisfactory for the red cell diluent, Toisson's solution causing lysis of the red cells. 2 p.c. oxalic acid solution was used to dilute the white cells. All counts were made in an improved Neubauer counting chamber. The hæmoglobin was estimated with a modified Sahli's apparatus with a glass scale, 100 p.c. on the scale being equivalent to 14 g. hæmoglobin per 100 c.c. blood. Six million red cells per c.mm. and 14 g. hæmoglobin per 100 c.c. of blood were taken as 100 p.c. normal, the colour index being calculated on this basis and not on the human scale. The mean cell diameter and the distribution curves were determined by the Price-Jones (1933) method.

Blood Picture in Normal Monkeys.

The literature contains few detailed references to the blood findings in normal monkeys. Scarborough (1931) from an analysis of the reported cases gives the following figures, but these refer to different species and not only to *M. rhesus*: red blood-cells, 5,590,000 per c.mm.; hæmoglobin, 75–103 p.c. on the Sahli scale (very few references); white blood-cells, 16,210 per c.mm.; no nucleated red cells in the peripheral blood and reticulocytes under 1 p.c., the reticulum being finer and staining more faintly than in man. The white cell-count is much more variable than in man, and in this respect monkeys resemble the smaller animals and children. The differential count is also very variable, but the polymorphonuclear cells are generally not more than 50 p.c. in absolutely normal animals and may be as low as 20 p.c. Scarborough also states that hæmolysis begins at 0·46 p.c. and is complete at 0·33 p.c. saline, a range very similar to that observed in human blood. Bilimoria (1931) working in Bombay gives the following figures for adult *rhesus* and *sinicus* monkeys: red blood-cells, 6,420,000 per c.mm.; hæmoglobin 91 p.c. (14 g. = 100 p.c.); white blood-cells, 18,129 per c.mm.; and mean cell diameter, determined by a halometer method, 6·4μ.

In the present work the animals were examined as they were received from the dealers and again while on control or stock diet. Unfortunately many of the animals on arrival were far from normal; some were suffering from generalized tuberculosis, many had heavy worm infections with an associated anæmia, and others were slightly anæmic, probably from faulty feeding and bad hygiene. The stools of the monkeys were examined in the Helminthology Department, through the courtesy of Prof. Leiper, and all were found to contain *Strongyloides fülleborni* and *Œsophagostomum apiostomum*, but when the infection was scanty the animals were apparently none the worse for it. After the sick and subnormal animals had been excluded there remained 13 judged to be healthy, and the observations recorded in Table I were obtained from these animals.

TABLE I.—*Observations on the Blood of* 13 *Normal* Rhesus *Monkeys.*

	Mean.	Number of observations.	S.E.*	C.V.†	Minimum value.	Maximum value.
Red blood-cells (millions)	6·23	113	0·599	9·6	5·08	7·81
Hæmoglobin p.c.	87	107	7·36	7·6	74	108
Colour index	0·84	107	..	..	0·80	0·93
M.C.D.‡	7·084	5	..	..	6·90	7·27
White blood-cells	15,240	116	4,364	28·6	8,200	28,700
Polymorphonuclear cells	41·5%	57	..	..	8%	75%
Lymphocytes	52%	57	..	..	22%	89%
Monocytes	2·4%	57	..	..	0%	8%
Eosinophils	2·1%	57	..	..	0%	8%

* = Standard error. † = Coefficient of variation. ‡ M.C.D. = Mean cell diameter.

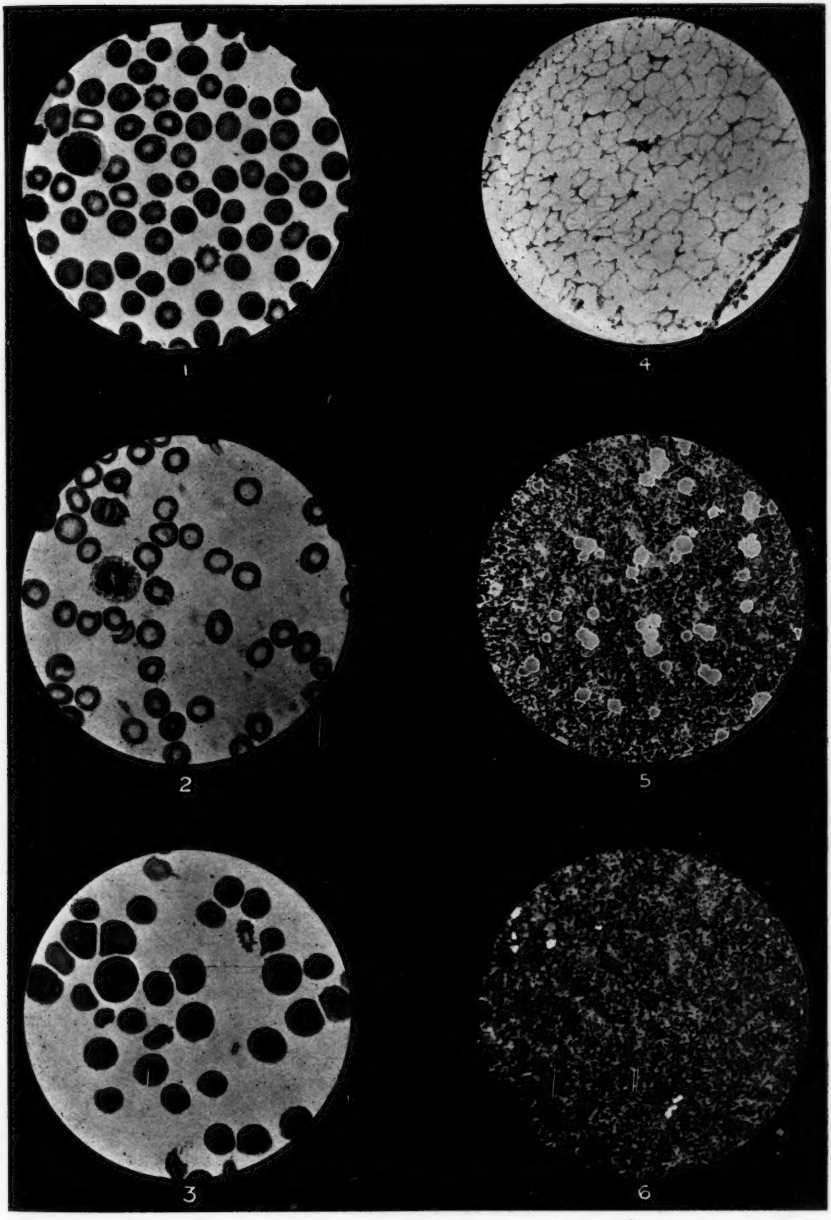
A study of the table shows that the average erythrocyte count was higher than that of man, though the hæmoglobin percentage was relatively lower. This should indicate, in the absence of an iron shortage, either that the cells were not fully saturated with hæmoglobin or that they were smaller than those of man. Further evidence pointed to the latter explanation as being correct; thus the mean cell diameter of 5 animals was 7·084μ, a value slightly lower than the figure 7·3μ generally taken as the mean value for man. It is possible that a larger series of observations would give a lower figure as the average for the mean cell diameter of normal monkeys. Further indication that the cells were fully saturated was the fact that an increased colour index was associated with an increase in the mean cell diameter (see later).

The fragility of the red cells was estimated in certain of these animals, and in all the normal monkeys hæmolysis began at 0·45 p.c. saline and was complete at 0·35 p.c. saline. In this respect and in all others investigated the red cells of the *rhesus* monkey resemble those of man very closely; even the average size, though slightly smaller than that of man, falls well within the normal human range.

When the white blood-cells were considered it was at once obvious that these differed both numerically and qualitatively from those of man. The average normal count was 15,000 per c.mm., and the variability of the count was very great. The percentage of polymorphonuclear cells was lower than the average human figure and the mature cell differed from the human type, frequently having very many lobes to the nucleus and finer granules in the cytoplasm. The lymphocytes were on the whole a little larger than the small

DESCRIPTION OF PLATES.

FIG. 1.—Blood smear, normal. × 570.
FIG. 2.— „ „ microcytic hypochromic anæmia. × 570.
FIG. 3.— „ „ macrocytic hyperchromic anæmia. × 570.
FIG. 4.—Bone-marrow section, normal. × 55.
FIG. 5.— „ „ „ microcytic hypochromic anæmia. × 55.
FIG. 6.— „ „ „ macrocytic hyperchromic anæmia. × 55.
FIG. 7.— „ „ „ normal. × 430.
FIG. 8.— „ „ „ microcytic hypochromic anæmia. × 430.
FIG. 9.— „ „ „ macrocytic hyperchromic anæmia. × 430.
FIG. 10.—Bone-marrow smears, normal. × 570.
FIG. 11.— „ „ „ microcytic hypochromic anæmia. × 570.
FIG. 12.— „ „ „ macrocytic hyperchromic anæmia. × 570.



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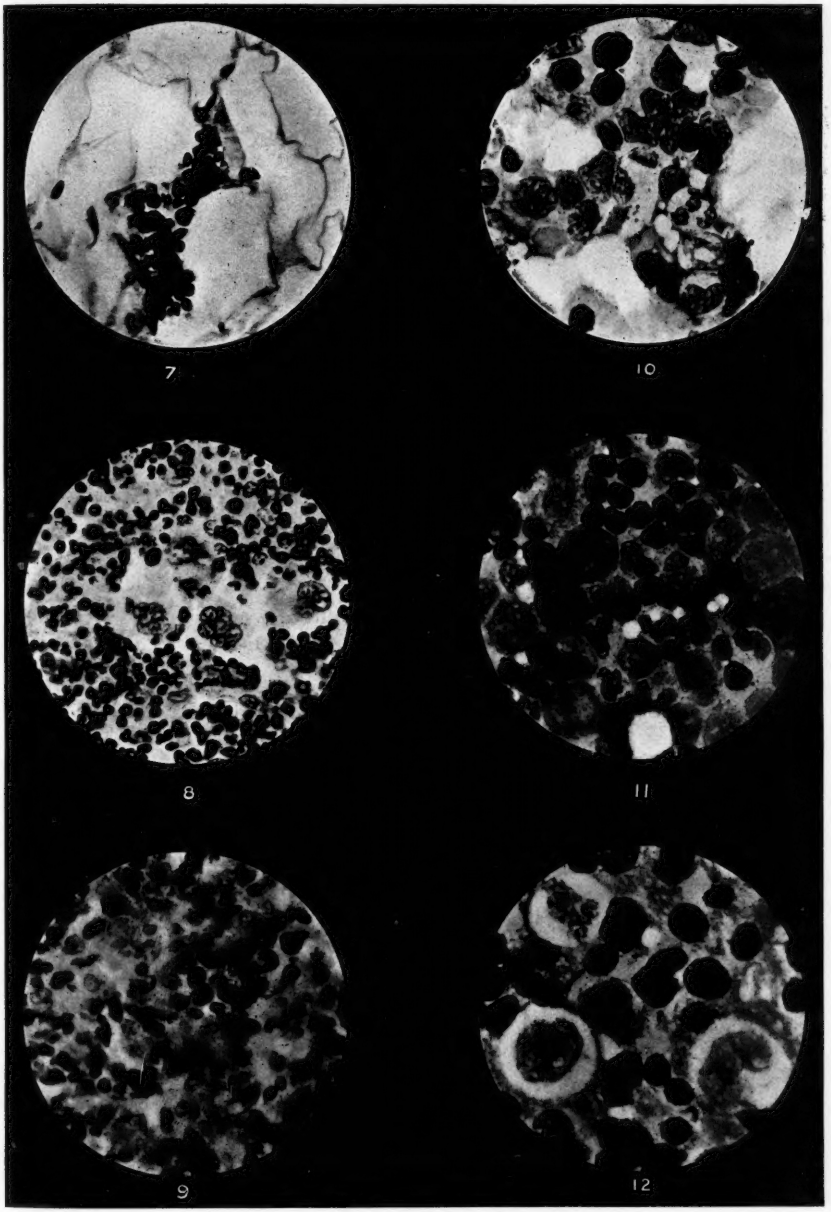


Wills and Stewart.

lymphocyte of man and the difference between the lymphocyte and the monocyte was not so marked.

Post-mortem material from normal animals was available from previous work and one control animal from the present series was sacrificed to obtain further material. The bone-marrow from the long bones (Figs. 4, 7, 10) was slightly more cellular than that of the adult human, but there were large numbers of fat-cells with small islands of cellular tissue between. These islands were composed largely of myelocytes and mature polymorphonuclear cells; there were a few normoblasts and myeloblasts. In all respects the marrow was very similar to the human type.

Microcytic, Hypochromic Anæmias.

The anæmia secondary to chronic hæmorrhage is taken as a typical example of this morphological class. In man this condition may at first show a normocytic or even a slightly macrocytic blood picture, but this rapidly gives place to that of a typical microcytic anæmia as the condition becomes chronic,

TABLE II.—*Blood Changes following Chronic Hæmorrhage in Various Species.*

	Red blood-cells.	Hæmo-globin p.c.	Colour index.	Duration of bleeding time in days.	Number of bleedings.	C.c. of blood removed.
Man*	4·78	95	1·00	..	..	..
Man‡	3·25	43	0·66	..	..	..
Monkey 5†	6·29	101	0·97	..	..	..
Monkey 5‡	3·57	39	0·60	30	9	260
Monkey 5, later	2·51	27	0·58	37	12	350
Monkey 7†	7·34	100	0·81	..	..	..
Monkey 7‡	1·67	12	0·43	67	15	380
Rabbit†§	5·14	69	0·67	..	..	..
Rabbit‡	3·61	48	0·67	40–122	18–24	480–730

* = Haden's figures (1932). † = Normal, before bleeding. ‡ = After chronic hæmorrhage.
§ = Steel's figures (1933), averages quoted.

TABLE III.—*Blood Changes in Chronic Hæmorrhage (Monkey No. 5).*

Date.	Red blood-cells.	Hæmo-globin p.c.	Colour index.	White blood-cells.	Reticulo-cytes.*	M.D.†	S.E.‡	C.V.§	Number of bleedings.	Total c.c. of blood removed.
3.10.34	6·29	100	0·95	11,200	0·9	7·57	0·57	7·5	0	0
18.10.34	4·73	71	0·91	15,900	8·1	..	..	..	2	58
31.10.34	3·35	46	0·83	11,400	7·3	..	..	..	5	148
14.11.34	3·57	39	0·70	8,600	9·5	7·09	0·65	9·36	9	258
21.11.34	2·51	27	9·64	8,500	..	6·57	0·75	11·5	12	348
26.11.34	2·77	29	0·63	11,600	6·2	..	..	..	13	370

* = Reticulocytes p.c. † = Mean diameter of red cells. ‡ = Standard error.
§ = Coefficient of variation.

the bone-marrow showing a normoblastic hyperplasia which varies in degree with the chronicity of the anæmia. In 2 monkeys subjected to repeated bleedings the response was in every way similar. This is shown clearly in Tables II and III, and in Figs. 2, 5, 8 and 11. At first there was a slightly increased red cell diameter, but after repeated bleedings there was a decrease

in the mean cell diameter and also a steady drop in the colour index. The increased percentage of reticulocytes was an index of the continuous effort at regeneration. *Post mortem* the depletion of the iron reserves of the body and the activity of the hæmopoietic system were well shown. There was marked pallor of all the organs and there was no increase in hæmosiderin in the tissues as shown by the ferrocyanide test on liver, spleen and kidney (macroscopic and microscopic tests). The bone-marrow of the long bones was red throughout the length of the shaft, and microscopically there was intense hyperplasia of the normoblastic type. The cells were chiefly myelocytes and mature granulocytes with islands of normoblasts amongst them; megakaryocytes were increased in number. Beyond these changes there was little of interest. It should be noted that there was no wasting, which is so characteristic a feature of nutritional macrocytic anæmia in monkeys.

A second example of a microcytic, hypochromic anæmia is the anæmia secondary to the chronic destruction of blood caused by repeated attacks of malaria. The animals used for this study were under Sir Rickard Christophers' care, and we were indebted to him for permission to make use of the material.

TABLE IV.—*Blood Changes after Repeated Attacks of Malaria. Record of Findings in Terminal Attacks (Monkey No. 16).*

Date.	Red blood-cells.	Hæmoglobin p.c.	Colour index.	White blood-cells.	Per 100 white blood-cells.		R'cytes.‡	M.D.§	S.E.‖	C.V.¶	Parasites.
					N'blasts.*	M'blasts.†					
10.7.33	4·84	..	..	6,400	6	0	1·0	..	..	..	++
18.7.33	5·11	60	0·71	9,400	..	..	..	6·73	0·67	9·9	0
21.7.33	5·61	68	0·72	..	..	..	3·5	..	..	..	0
24.7.33	3·04	..	..	..	..	..	..	7·3	0·64	8·6	0
27.7.33	2·90	38	0·77	..	0	0	3·5	7·3	0·67	9·1	0
29.7.33	4·25	52	0·74	13,500	4	0	19·0	..	..	..	0
9.8.33	3·33	..	..	16,800	15	0	..	..	..	..	++
10.8.33	1·66	21	0·75	..	33	0	6·0	6·52	0·83	12·8	+
11.8.33	1·43	18	0·75	21,400	18	0	14·2	6·30	0·76	12·1	+

* = Normoblasts. † = Megaloblasts. ‡ = Reticulocytes. § = Mean diameter of red-blood cells.
‖ = Standard error. ¶ = Coefficient of variation.

The monkeys were all infected experimentally with *Plasmodium knowlesi*, a chronic infection being obtained by treating with sub-maximal doses of atebrin. This enabled the animals to survive the attacks without being cured. The results of a typical experiment are shown in Table IV. During the first few attacks the animal developed a moderate degree of anæmia of the normocytic type which improved after each dose of atebrin; this type of anæmia is that usually seen in human malaria (Fairley and Bromfield, 1934). Then, as the attacks continued, the anæmia became more severe and microcytic in type, the mean cell diameter having fallen to 6·3μ by the end of the experiment. The colour index was moderately decreased but remained very constant throughout. (Values obtained after atebrin were excluded, as this drug colours the serum.) The blood picture was similar to that of human malaria and showed moderate anisocytosis and poikilocytosis with the marked

polychromasia which is such a characteristic feature of the blood picture in man. The polychromasia and the increased number of reticulocytes and circulating normoblasts were an index of the persistent effort at regeneration. As each relapse occurred large numbers of parasites appeared in the blood.

The post-mortem findings were characteristic of chronic malaria: the most noteworthy changes being the enlarged, almost black spleen and a liver showing similar changes to a lesser degree. Microscopically the sections of spleen, liver and kidneys were seen to contain large deposits of the malarial pigment, especially in the splenic pulp, with smaller amounts of hæmosiderin in the same organs. Ziemenn (1934) describes a very similar condition in chronic malaria and the sections shown in a coloured plate (p. 212) resemble those from the monkeys very closely. The bone-marrow of the long bones was red from end to end of the shaft and microscopically the hyperplasia was seen to be normoblastic. There was a large amount of malarial pigment in the marrow, some free and some in large phagocytic cells. In certain animals the red cells contained numerous parasites, in others few were found, the findings depending on the stage in the malarial cycle in which the animal died.

Macrocytic Hyperchromic Anæmia.

This anæmia was produced by feeding the monkeys on a diet based on one in common use among the poorer class Mohammedans in Bombay (Wills and Talpade, 1930), who suffer more severely than any other community in the city from tropical macrocytic anæmia. Details of the diet are given in Table V. This diet was extremely low in protein, and contained none of animal

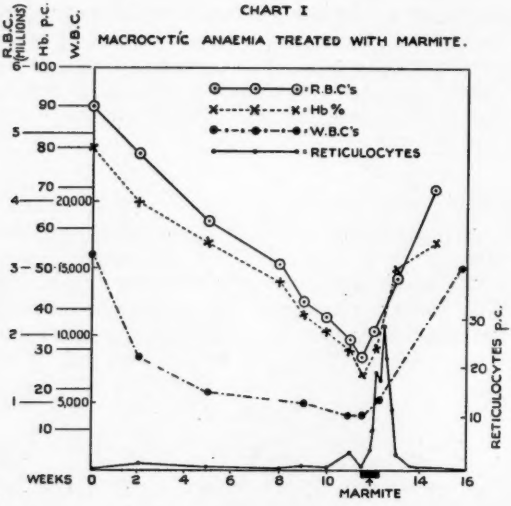
TABLE V.

Daily ration.	Parts.	Preparation.
Polished rice	40	Cooked together by boiling till rice is soft.
Margarine (to replace ghi)	15	
Common salt	to flavour	
White bread	45	Mixed with the above after cooking.
Cod-liver oil	3 c.c. per animal	
Tomato or carrot	25 g. ,,	Given separately.

origin, and though supplemented as regards vitamins A, C and D, it was very low in the vitamin B complex, owing to the poor quality of the cereals used and the absence of meat, liver or yeast from the diet. Salts other than sodium chloride were also deficient. Each monkey was fed with an excess of the mixture. The rations of cod-liver oil and fresh vegetables were sufficient to prevent rickets and scurvy, and with a few exceptions the monkeys suffered from no intercurrent infections. Part way through the experiment some of the animals showed signs of an iron deficiency, so iron, as iron and ammonium citrate $\frac{1}{2}$ g. daily per animal, was added to the diet to prevent this complication. A ration containing about 70 g. rice, 80 g. bread and 17 g. margarine was

adequate to satisfy appetite and allow for wastage. Two monkeys were kept as controls. These received exactly the same diet with the sole addition of 5 g. of marmite daily. This amount of marmite is in excess of basic requirements, but as it was mixed with the food it was necessary to give excess to allow for wastage.

The experimental animals remained well at first and then as the anæmia developed they lost weight, became inactive, tended to walk and sit with feet tightly clenched, and lost the hair from the tail. In the later stages of the anæmia there was definite œdema, which varied in intensity from case to case. The œdema was more than that associated with any severe anæmia and was apparently related to a vitamin B_1 deficiency, as it improved, with



no corresponding improvement in the anæmia, on the addition of small quantities of dried yeast to the diet. The same type of œdema was seen in some cases of tropical macrocytic anæmia in Madras, where vitamin B_1 deficiencies are common. Only terminally did the monkeys lose their appetite and refuse food. The time taken for the anæmia to develop varied within wide limits from animal to animal, the shortest time was 3 and the longest 9 months. The control animals remained well for 18 months, when one was killed for post-mortem material and the other put on the experimental diet.

The blood changes in typical examples of this anæmia are set out in Table VI and on Chart 1, and for purposes of comparison the average figures from a series of cases of tropical macrocytic anæmia observed by the senior author (Wills and Mehta, 1930) are included in the table. The anæmia was both macrocytic and megaloblastic: the colour index rose as the anæmia increased in severity, showing that there was no shortage of iron. The degree of anisocytosis was less than in true pernicious anæmia, in other words the

TABLE VI.—*Blood Changes in Macrocytic Hyperchromic Anæmia (Monkey No. 4).*

Date.	Red blood-cells.	Hæmo-globin p.c.	Colour index.	White blood-cells.	Per 100 white cells counted.		R'cytes.*	M.D.†	S.E.‡	C.V.§
					Normo-blasts.	Megalo-blasts.				
15.8.33	6·15	87	0·87	15,500	0	0	0·4	6·86	0·59	8·6
11.12.33	4·45	72	0·98	..	1	..	1·5	8·01	0·76	9·5
20.3.34	6·62	88	0·89	24,000	0	0	1·9	..	..	..
25.6.34	4·99	70	0·84	14,700	0	0	0·4	..	..	..
5.9.34	3·97	61	0·92	..	0	0	0·6	7·67	0·48	6·3
8.10.34	3·49	56	0·97	5,000	0	0	0·6	8·11	0·65	8·0
21.10.34	3·26	55	1·02	..	16	10	..	..	..	..
24.10.34	2·23	40	1·08	3,600	130	6	..	8·01	0·91	11·3

Between December, 1933, and March, 1934, the animal received treatment.

Average figures for 50 untreated cases of tropical macrocytic anæmia:

..	0·97	20	1·12	7,394‖	2·6	1·5	8·41¶	1·01¶	12·0¶

* Reticulocytes p.c. † = Mean diameter of red blood-cells. ‡ = Standard error. § = Coefficient of variation. ‖ = Many pregnant cases included in series, the average figure for non-pregnant cases was 4,000. ¶ = Average from 20 cases.

coefficient of variation of the red cells was smaller; this was so in both the human and the monkey cases. Megaloblasts were constantly present in the blood at the height of the anæmia, but, both in the terminal stages and during active regeneration, very numerous normoblasts as well as megaloblasts were seen. This is also true of the human cases. In one monkey there was a true blood crisis terminally, when for every 100 white cells counted there were 130 normoblasts and 6 megaloblasts. As the anæmia developed there was a significant fall in the number of leucocytes, white cell-counts as low as 4000 being not uncommon. In treated cases the white cells increased again but more slowly than the red cells.

In all cases in which the Van den Bergh test was applied both direct and indirect reactions were negative. Further evidence against an increased blood destruction was the fact that when tested at the height of the anæmia the range of hæmolysis was found to have shifted to the left, beginning at 0·35 p.c. saline and being complete at 0·2 p.c.

The post-mortem findings differed from those seen in cases of idiopathic pernicious anæmia: as post-mortems were never obtained in the author's series of cases of tropical macrocytic anæmia it was impossible to make any comparison with that condition. On section the most striking change was the almost complete absence of fat throughout the body. None of the organs showed gross macroscopic changes except the bone-marrow. In the long bones this was red and gelatinous from end to end and obviously in a condition of marked hyperplasia. Microscopically (Figs. 6, 9, 12) the marrow showed a megaloblastic reaction. In addition there were masses of large, pale, non-granular primitive cells with very variable shaped large pale nuclei, some of which showed mitotic figures; these cells were relatively enormous and formed the most conspicuous feature in some of the sections and smears (Fig. 12). The liver and spleen appeared normal macroscopically, but sections

showed deposits of hæmosiderin in the liver-cells, especially at the periphery of the lobule, round the portal vein and bile capillaries; in the spleen similar deposits were seen throughout the pulp, in marked contrast to the Malpighian corpuscles, which contained none. Unlike cases of pernicious anæmia these monkeys showed no fatty degeneration in the liver or heart muscle: actually there was less fatty change in the liver-cells than in those of the control and normal animals.

The curative action of marmite was demonstrated in several cases, and Chart 1 shows the result of such treatment in a typical case. The erythrocyte count had fallen from 5·40 millions to 1·67 millions, when a maximal response, as shown by the reticulocyte crisis, followed the administration of 20 g. of marmite on 5 consecutive days, and within 20 days the count had risen over 2·5 millions. This response is typical of that seen in cases of tropical macrocytic anæmia treated with marmite and cases of idiopathic pernicious anæmia treated with liver extracts by the mouth. The blood crisis resulting from this treatment was very striking in this particular monkey, an enormous number of nucleated red cells of all types appearing in the peripheral circulation. An exactly similar picture was sometimes seen in the human cases. In previous experiments (Wills and Bilimoria, 1932) monkeys suffering from the same anæmia responded equally well to liver extract by the mouth.

Discussion.

The foregoing experiments show that, within the field of inquiry, the hæmopoietic system of the *rhesus* monkey behaves in a manner remarkably similar to that of man, and therefore that the animal is a suitable subject for the experimental study of anæmias, particularly macrocytic anæmias. The only finding needing discussion as differing from the picture in human anæmia is the bone-marrow hypertrophy in the macrocytic anæmia in monkeys. The megaloblastic reaction was very similar to that seen in pernicious and other human macrocytic anæmias, but the large, pale primitive cells, which were present in such large numbers in the marrow from some of the animals, are not commonly seen in preparations from human material. These cells were found in greatest number in the bone-marrow from the monkey that died during a blood crisis, during which very numerous nucleated red cells appeared in the blood-stream, and they would appear to be stem-cells, though their relation to the red and white cell series could not be determined from our preparations.*

No consideration of macrocytic anæmia in animals should fail to include the possibility of a *Bartonella* infection. Thus a macrocytic anæmia has been produced in intact rats on deficient diets. This anæmia, however, was accompanied by the appearance of *Bartonella* bodies in the circulating blood, and, whilst many animals died in the acute stage of the illness, those that survived made a spontaneous recovery, the anæmia disappearing without any specific treatment (Wills and Mehta, 1930). Recently also Miller and Rhoads (1935), who have induced a macrocytic anæmia in dogs by faulty feeding,

* Since the above was written Prof. Turnbull has very kindly examined the bone-marrow from this monkey and identifies these cells as leucoblastic cells of the neutrophil series.

have reported the finding of *Bartonella canis* in their experimental cases after splenectomy. The exact interpretation of these findings is at present obscure, as the *Bartonella* bodies were never found in non-splenectomized animals fed on the same faulty diet, even after they had received injections of blood from the infected animals. Moreover, the infected animals could be cured of their anæmia, with the disappearance of the *Bartonella* bodies, by adding lean meat to the diet.

The question whether a latent *Bartonella* infection is present in the monkeys has not been determined and must remain unsettled until sufficient animals are available for a series of splenectomies to be done. Should, however, all the monkeys prove to be carriers of *Bartonella* it would not invalidate the work described above, as the animals behave in every way like man under similar circumstances, but the finding of *Bartonella* bodies would open up further fields of research into the effect of diet upon immunity to such infections and conversely the influence of such infections on the response of an animal to dietetic deficiencies.

We are indebted to Dr. Dorothy Vaux for help with the post-mortems and histological material, and to Mr. M. C. Luck for technical assistance with the microphotographs.

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BACT. AERTRYCKE ENDOTOXIN: A STUDY ON ITS PRODUCTION, DETOXICATION AND THE PRACTICAL UTILIZATION OF ITS ANTIGENIC PROPERTIES.

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THE subject of food infection due to *Bact. aertrycke* has hitherto received but little experimental attention in South Africa, probably as a result of the rarity of outbreaks among humans there. Interest, however, was focused upon the problem by a severe outbreak which occurred on a train during 1932. The human pathogenicity of the strain was strikingly demonstrated by the acute nature of the intoxication, which was responsible for 18 cases and one death, and its immunological characters warranted intensive study, because such an investigation supplemented the researches on typhoid, paratyphoid and other intestinal infections which had been carried on in the Serum Department of this Institute over a period of years. The results of our work on endotoxins, endotoxoids and the production of specific antisera applied to the prophylaxis and treatment of these conditions was so encouraging that this course of investigation was pursued in preference to lines upon which numerous workers in other countries are now concentrating. Although our studies on *Bact. aertrycke* have now advanced far enough to enable us to undertake fractionation experiments, our original aim was not to prepare specific fractions in a more or less pure state, but an antigen which could readily be produced in sufficient quantities to be of practical use in the production of a vaccine and of a specific therapeutic serum. Briefly, we set out to ascertain whether our strain would give rise to a potent endotoxin, whether such an endotoxin could be rendered innocuous, whether it possessed antigenic properties and whether the information afforded could be put to practical use should the necessity again arise.

VIRULENCE AND TOXICITY OF THE STRAIN.

Virulence.

The cultural, morphological and biochemical properties of the strain had been exhaustively dealt with by the Bacteriological Department of the Institute, so that our first object was to obtain a preliminary assessment of its virulence for laboratory animals.

Eighteen-hour agar cultures were emulsified and diluted in normal saline so that the final concentration was 12,000 million organisms per c.c. Intraperitoneal injection of decreasing quantities of such emulsions showed that the minimum fatal dose for rats was 0·5 c.c., and for mice 0·05 c.c. Subsequent

examination showed that the strain as received consisted of a mixture of typically rough and smooth colonies ; these forms were isolated in pure culture and by regular subculture and storage in the dark they proved to be exceedingly stable, there being but little tendency to reversion. Comparative tests with the two forms revealed that the virulence of the smooth culture was approximately 3 to 4 times that of the rough.

The Toxic Properties of the Strain.

Numerous toxic fractions have been isolated form *Bact. aertrycke* in a variety of ways, some of the most recent work on various aspects of the subject having been carried out by Raistrick and Topley (1934), Delafield (1934), Martin (1934) and Boivin, Mesrobeanu, L., and Mesrobeanu, I. (1934).

In attempting to procure a potent *Bact. aertrycke* endotoxin, however, we considered it logical to apply the method of repeated alternate freezing and thawing, which had afforded such gratifying results in the conditions already mentioned (Grasset and Gory, 1927 ; Grasset, 1930, 1935). Autolysates produced in this manner are apparently much more stable than those obtained by Rowland with liquid air, the toxic and antigenic properties of the latter having proved to be very unstable, possibly as the result of oxidation.

Roux bottles of agar were employed, care being taken to pipette off all the water of condensation in order to eliminate those extraneous fractions which are known to give rise to non-specific reactions. The bottles were inoculated with *Bact. aertrycke* and incubated for 20 hours, the resulting growth being washed off in normal saline so that 10 bottles provided 100 c.c. of emulsion, which was killed by heating to 60° C. for one hour. The sterile emulsions were then frozen hard in a specially designed freezing unit at a temperature of —30° C. for 6 successive nights, thawing taking place during the day at laboratory temperature. The lysed emulsion was then centrifuged at high speed for 30 minutes.

The average protein content of the original emulsions was 1·1 p.c. ; centrifugation resulted in a limpid supernatant endotoxin with a protein content of 0·6 p.c.

Toxicity of the Autolysates.

The animals used in assessing the toxicity of the autolysates comprised mice, rats, rabbits, sheep, goats, pigs, donkeys and horses, and fowls were also employed regularly because of the controversial nature of the literature on this subject relating to them.

The fatal doses listed in the Table have been compiled from a very large number of tests ; they cause death in from 16 to 24 hours, during which period a progressive course of symptoms may be observed, typical of a true intoxication and free from any semblance of shock.

All our attempts at producing death by other than parenteral means proved unsuccessful, an experience common to most workers in this field. In addition to experimentation on laboratory animals and birds, a number of sucking pigs were utilized for this purpose, it being considered that these might hold out some prospect of success. Massive doses of killed emulsion or potent

TABLE I.—*Potency of the Endotoxin Derived by Repeated Freezing and Thawing of Killed Emulsions of* Bact. aertrycke.

	Route.	Fatal dose of endotoxin, c.c.
Mouse	Subcutaneous	0·15
,,	Intraperitoneal	0·05
Rat	Subcutaneous	3·00
,,	Intraperitoneal	0·15
Guinea-pig	Subcutaneous	4·00
,,	Intraperitoneal	0·75
Rabbit	Intravenous	0·15
,,	Subcutaneous	4·00
,,	Intraperitoneal	0·80
Sheep	Intravenous	5·00
Goat	,,	10·00
Horse	,,	10·00
Ass	,,	10·00
Fowl	,,	1·00*
Pig	Intraperitoneal	8·00

* It is of interest to note that pigeons were able to withstand 5 c.c. endotoxin intravenously without obvious ill effects, and in no case were we able to produce more than moderately severe toxic symptoms. Fowls, on the other hand, were killed by 1 c.c. endotoxin, and although they undoubtedly possess a somewhat high resistance, our results are contrary to the experience of Cameron and Rettger (1934) who found that alternate freezing and thawing failed to liberate demonstrable *aertrycke* toxin for fowls.

endotoxin were administered *per os*, *per rectum*, in gelatin capsules and as keratin-coated pills, with and without previous administration of ox bile, but in every case the animals survived without apparent ill effects.

DETOXICATION OF POTENT *BACT. AERTRYCKE* AUTOLYSATES.

The previously quoted papers on typhoid endotoxin show that highly potent typhoid autolysates may be rendered atoxic at 37° C. by means of formalin in approximately one month, the resulting product being antigenic and suitable for the production of either therapeutic sera or a prophylactic soluble vaccine (typhoid endotoxoid). An analogous procedure was followed in the case of the *Bact. aertrycke* autolysates; successive batches were divided up into convenient quantities to which varying proportions of formalin were added, the autolysates thus treated being placed at incubator temperature and tested for toxicity at regular intervals.

It was soon evident that aertrycke autolysates would require a somewhat longer and more vigorous course of treatment than was necessary in the case of typhoid autolysates. Rats and mice injected intraperitoneally with the equivalent of 5 fatal doses of autolysate which had been incubated for one month in the presence of 0·4 p.c. formalin died in a few hours. Prolonging the period of incubation to 50 days failed to afford a completely atoxic product, for animals surviving the injection of 5 previously fatal doses died from the toxic effects of 10 such doses with but little delay as compared with controls.

They were able, however, to withstand subcutaneous injections of 20 previously fatal doses without apparent ill effects.

Further work on detoxication tended to show that an increase in the proportion of formalin was more effective than prolonging the period of incubation, and, since this is also the more satisfactory alternative from the practical viewpoint, the method of detoxication finally decided upon for the average batch of autolysate was 6 weeks incubation at 37° C. in the presence of 0·6 p.c. formalin. Occasional batches of unusually high toxicity may require somewhat longer incubation, but as a general rule one obtains a product at the end of 6 weeks which can be used safely for the immunization of small animals, and, as we shall show, for the hyperimmunization of horses.

ANTIGENIC PROPERTIES OF THE DETOXICATED AUTOLYSATES.

Having thus succeeded in rendering the highly toxic *aertrycke* autolysates innocuous, we were able to resort to the immunization of laboratory animals in order to ascertain whether a course of 3 or 4 subcutaneous injections would enable them to withstand significant test injections of live cultures. Rats and mice were chosen for the purpose, and the following experiments show the nature of the immunization and the degree of response.

Immunization of Mice with Detoxicated Aertrycke *Autolysates.*

White mice received subcutaneous injections of 0·5 c.c., 1 c.c. and 2 c.c. detoxicated autolysate with an interval of 12 days between injections. The immunized mice when tested intraperitoneally with graded doses of live virulent culture were able to withstand as much as 8 fatal doses. Since a total of only 3·5 c.c. antigen was administered, the results must be considered as eminently satisfactory and a definite indication of the antigenic value of the autolysates used.

Immunization of Rats.

Group 1.—The white rats in this group received a course of 3 subcutaneous injections of detoxicated autolysate consisting of 1 c.c., 2 c.c. and 3 c.c. Twelve days after the last immunizing dose the animals were able to withstand as much as 3 fatal doses of live culture intraperitoneally.

Group 2.—In order to ascertain whether an increase in the amount of autolysate administered would give rise to a corresponding increase in the immunity response, this group received the same course of injections as the rats in Group 1, plus a fourth immunizing dose of 4 c.c. These animals were able to resist more than 4 fatal doses of live culture ; the degree of response is thus largely influenced by the intensiveness of the course of immunization.

Group 3.—Although from a practical point of view it had seemed advisable to carry out the major portion of this investigation with the strain of *Bact. aertrycke* in the form in which it was originally isolated, a considerable amount of comparative work on the smooth and rough forms was also performed. Since we had already seen that the virulence of the smooth form was regularly 3 to 4 times that of the rough and that it gave rise to an endotoxin of very much greater potency, it was a point of interest to investigate the antigenic properties

of the two forms. The rats in Group 3 were therefore divided into two series, one receiving a course of immunization with detoxicated autolysate derived from the smooth form and the other with an exactly similar antigen derived from the rough form. When the rats were tested by intraperitoneal injections of live cultures, the resistance of those immunized with "smooth" antigen was more than twice that of those which had received a similar course of injections of "rough" antigen.

HYPERIMMUNIZATION OF HORSES.

The results afforded by rats and mice indicated that the principle might be advantageously applied to the hyperimmunization of horses. Apart from the possibility of obtaining an immune serum of therapeutic value, the study of phenomena such as agglutination, flocculation and neutralizing properties *in vivo* and *in vitro* promised to be of interest and value.

Horse 1 received a series of 7 subcutaneous injections of detoxicated *aertrycke* autolysate at intervals of 10 days; the doses consisted of 10 c.c., 20 c.c., 25 c.c., 30 c.c., 50 c.c., 75 c.c. and 100 c.c. Samples of blood were taken immediately before each injection and a bulk bleeding was carried out 10 days after the last injection. After a period of rest the horse received a re-immunization consisting of 6 injections of 40 c.c., 75 c.c., 100 c.c., 135 c.c, 175 c.c and 200 c.c. Horse 2 received a somewhat more vigorous course of immunization; the doses were spaced as before but consisted of 9 injections totalling 800 c.c.

Reaction of Horses to Immunization.

Despite the fact that the autolysates employed were sufficiently atoxic to enable mice to withstand intraperitoneal injections of the equivalent of 5 fatal doses, the horses showed consistently big reactions after each injection; these took the form of a rise in temperature to 105° F., local œdema and acute systemic disturbances. Before returning to normal the animals were depressed, there was a shrinkage of muscular elements and occasional diarrhœa.

Agglutination.

Agglutination tests were carried out on each sample of serum and on the final bleedings; live emulsions were employed, the reactions taking place at incubator temperature. The limit of complete agglutination increased progressively with each injection, the first giving rise to a titre of 1 in 350, this increasing regularly until a titre of 1 in 20,000 was obtained with the serum of the bulk bleeding. The reactions were typical and specific, no agglutination occurring when other bacterial suspensions were used in the place of *Bact. aertrycke.*

Flocculation.

The flocculation reaction exhibited by anti-typhoid serum produced by hyperimmunizing horses with detoxicated typhoid endotoxin has been closely studied during the last few years and has been the subject for a publication by one of us (E. Grasset, 1934). The phenomenon is characteristic and specific: it may be used to provide a fairly accurate assessment of both the neutralizing

value of a serum and of the soluble typhoid antigen in either its toxic or detoxicated form.

Each sample of serum obtained from horses undergoing the course of immunization with *aertrycke* autolysate was submitted to the flocculation test; selected batches of endotoxin prepared as previously described, were spun at very high speed until the supernatant liquid was quite limpid, the serum being similarly treated. Varying volumes of serum were added to constant volumes of the endotoxin, the mixtures being thoroughly shaken and left, usually, at laboratory temperature, although for comparative purposes the tests were sometimes carried out at 37° C.

Taken as a whole the reactions are not as characteristic as those regularly obtained with typhoid serum; after a period of approximately 15 minutes a zone of opalescence appears followed about 2 hours later by an inter-action more in the nature of a granular precipitation than a flocculation. Nevertheless, the phenomenon is distinct and definite, its specificity being easily proved by carrying out parallel tests with typhoid and other sera produced on the same principle.

The results of the flocculation tests may be summarized as follows: after the first injection of the horse, 2·4 c.c. serum produced the first sign of initial flocculation with 1 c.c. endotoxin in 1½ hours at laboratory temperature; this volume of serum decreased with each successive injection until at the end of the immunization 0·4 c.c. serum gave rise to initial flocculation. It is interesting to note that although the volume of serum becomes progressively smaller as the immunization proceeds, the time of flocculation becomes prolonged to as much as 4 hours in some cases.

Neutralizing Properties of the Serum.

The neutralizing properties of the serum were assessed by the intraperitoneal injection of mice, the object being to ascertain the number of fatal doses of endotoxin which could be neutralized by 1 c.c. of serum. Mixtures of 1 c.c. serum and progressively increasing quantities of endotoxin were allowed to remain in contact for one hour at laboratory temperature. It was found that the neutralizing value of the serum gradually increased until at the end of the hyperimmunization mice were able to withstand the injection of mixtures of 1 c.c. serum and as much as 7 fatal doses of endotoxin, a very gratifying result when compared with a neutralization of from 10 to 15 fatal doses afforded by typhoid sera of definitely high therapeutic value.

We are at present engaged on the problem of concentrating and purifying the *aertrycke* serum on lines which have proved satisfactory when applied to typhoid and paratyphoid sera and antivenene. Should the method prove to be applicable, it is hoped that a specific antiserum of considerable value will be available for therapeutic use in the event of other outbreaks of food infection due to *Bact. aertrycke* occurring in South Africa.

SUMMARY.

1. A recently isolated and highly virulent strain of *Bact. aertrycke* responsible for an acute outbreak of food infection on a train has been the subject of this study.

2. A potent endotoxin was obtained by alternate freezing and thawing of concentrated killed emulsions.

3. The toxic autolysates were rendered atoxic by the action of 0·6 p.c. formalin in the course of 6 weeks incubation at 37° C.

4. The antigenic properties of the detoxicated autolysates have been demonstrated by the immunization of mice and rats.

5. The hyperimmunization of horses gave rise to sera possessing specific and high agglutinating properties, flocculating properties and considerable antibacterial and antitoxic values as proved by tests *in vivo* and *in vitro*.

6. The concentration and refinement of the horse-serum is now being investigated.

7. The investigation tends to show that the *aertrycke* antiserum may be utilized with satisfactory therapeutic results should the necessity arise.

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THE EFFECT OF CERTAIN CONSTITUENTS OF CULTURE MEDIUM ON THE PRODUCTION OF DIPHTHERIA ANTITOXIN IN NORMAL AND IMMUNIZED GUINEA-PIGS.

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In previous papers (Hartley, 1925, 1926) it has been shown that by making use of Ramon's flocculation reaction over 99 p.c. of the nitrogenous impurities occurring in toxin-antitoxin mixtures can be removed, and that the final product obtained—toxin-antitoxin floccules, a white precipitate insoluble in normal salt solution—is a very effective antigen. The preparation, properties and antigenic activity of a number of such purified diphtheria antigens, separated from mixtures of different composition, have been described. During the course of this earlier work it was frequently observed that groups of animals which received a certain dose of purified antigen produced more antitoxin than similar groups which received the whole mixture of toxin and antitoxin from

which the purified antigen had been prepared. These and other observations suggested that by means of purified antigens of this type, it would be possible to study the effect of the impurities occurring in diphtheria toxin and diphtheria prophylactic on the activity of the specific antigen and the production of antitoxin. In these earlier experiments the antigen was injected, in a total volume of 2 c.c., in normal salt solution ; it seemed therefore that by injecting the same dose of purified antigen emulsified in solutions containing certain of the impurities occurring in diphtheria prophylactic, it might be possible to determine whether any of these affected the production of antitoxin.

EXPERIMENTAL.

A. *Diphtheria Antitoxin Production in Normal Guinea-pigs. The Effect of Nutrient Broth and the Constituents thereof, and of other Substances, on the Activity of a Purified Diphtheria Antigen (Toxin-Antitoxin Floccules).*

Experiment 1.

The purified antigen used in this experiment was prepared in the following way :

A neutral (L_0) mixture of toxin and antitoxin was made by adding 5 c.c. of diphtheria antitoxin (500 units per c.c.) to 250 c.c. of diphtheria toxin (L_0 dose = 0·10 c.c.). Flocculation occurred in about 3 hours and a small amount of precipitate settled to the bottom of the cylinder. The mixture was allowed to stand at room temperature in the dark for 4 days. The clear supernatant fluid was then siphoned off and the precipitate washed 4 times with 250 c.c. of normal salt solution. The white insoluble precipitate was finally emulsified in 25 c.c. of normal salt solution. This constituted the purified antigen A, used in the first series of experiments.

The following mixtures were then prepared :

(1) 2 c.c. antigen A + 18 c.c. normal salt solution.
(2) „ „ „ phosphate buffer solution pH = 9.
(3) „ „ „ phosphate buffer solution pH = 5.
(4) „ „ „ 0·5 p.c. carbolic acid in normal salt solution.
(5) „ „ „ 0·1 p.c. formalin in normal salt solution.
(6) „ „ „ digest broth (Hartley, 1922).
(7) „ „ „ Difco broth (Hartley and Hartley, 1922).
(8) „ „ „ muscle extract.
(9) „ „ „ 2 p.c. Difco proteose peptone in normal salt solution.

The mixtures were proved to be sterile, and the reaction of each was determined. After standing at room temperature for 4 days the mixtures were inoculated into guinea-pigs. (The guinea-pigs used in all the experiments described in this paper were drawn from the same stock, reared at Rhodes Farm and supplied for use at Hampstead. 168 guinea-pigs were used in the experiments here recorded ; none died during the course of the experiments and all increased in weight.) Six animals were used for the study of each mixture and each guinea-pig was injected with 2 c.c. quantities, *i. e.* 0·2 c.c. of purified antigen A, + 1·8 c.c. of diluent. The animals used were of corresponding age and weight throughout the series, and were kept under the same conditions as regards diet and housing. 74 to 78 days after injection the animals were bled and the antitoxin content of the serum determined by the intracutaneous method.

The results of this experiment (Table I) suggest that faintly acid solutions and carbolic acid adversely affect the activity of the antigen, but further experiments on larger groups of animals are necessary in order definitely to establish whether this is the case. It may be suggested, however, that if exposure of the antigen to 0·5 p.c. carbolic acid for only 4 days affects its

TABLE I.

A = antigen = washed floccules from a neutral diphtheria toxin-antitoxin mixture (0·2 c.c. of the floccules emulsion contained 0·023 mg. nitrogen).

Series.	Injected with—	pH.	Bled after days.	Antitoxin produced: units per c.c. serum.					
1	0·2 c.c. A + 1·8 c.c. saline	7·0	74	0·2,	0·2,	0·15,	0·1,	0·1,	0·1
2	0·2 c.c. A + 1·8 c.c. alkaline buffer soln.	9·2	75	0·2,	0·2,	0·15,	0·15,	0·1,	0·04
3	0·2 c.c. A + 1·8 c.c. acid buffer soln.	5·0	75	0·1,	0·1,	0·04,	0·02,	0·02,	0·002
4	0·2 c.c. A + 1·8 c.c. 0·5 p.c. carbol saline	7·4	77	0·08,	0·06,	0·06,	0·04,	0·04,	0·04
5	0·2 c.c. A + 1·8 c.c. 0·1 p.c. formol saline	7·2	77	0·15,	0·15,	0·15,	0·1,	0·1,	0·04
6	0·2 c.c. A + 1·8 c.c. digest broth	7·4	77	0·08,	0·06,	0·04,	0·02,	0·005,	0·005
7	0·2 c.c. A + 1·8 c.c. "Difco" broth	7·4	77	0·06,	0·02,	0·02,	0·01,	<0·005,	<0·005
8	0·2 c.c. A + 1·8 c.c. meat extract	7·2	78	0·2,	0·1,	0·04,	0·02,	0·02,	0·02
9	0·2 c.c. A + 1·8 c.c. "Difco" peptone	7·4	78	0·2,	0·2,	0·2,	0·1,	0·1,	0·1

activity to the extent indicated in the protocol, then, in view of the common use of this substance as a preservative, the effect of exposure of diphtheria antigens of this type to the action of carbolic acid for longer periods merits further investigation. In the present series of experiments attention has been directed more especially to the action of peptone and muscle extract on the activity of toxin-antitoxin floccule preparations. The results of this first experiment confirm the earlier observations in so far as they suggest that antigenic activity is unfavourably affected by ordinary nutrient broth, and, moreover, that this detrimental action is due more to the ingredients furnished by the muscle extract than to those supplied by the peptone.

Experiment 2.

This was carried out to investigate further the action of muscle extracts and peptones on the activity of a purified antigen.

Four muscle extracts were prepared from beef, veal, horse muscle and guinea-pig muscle. In each case the muscle was cleaned from fat and finely minced. Tap-water was added at the rate of 1 l. per 500 g. of minced muscle and the muscle-water mixture allowed to stand in the cold room overnight. It was then steamed for 1 hour, filtered through paper, the reaction of the filtrate adjusted to pH 7·6, filtered again, and the clear filtrate finally sterilized by heating to 110° C. for half an hour.

2 p.c. solutions of Parke Davis peptone, Difco proteose peptone and Witte's peptone in normal salt solution were also prepared. After the reactions had been adjusted to pH 7·6, the solutions were filtered, and sterilized by heating to 110° for half an hour. Two other preparations were included in this series. These were "peptones" prepared by the writer, one by the action of pepsin on horse-serum albumin, and the other by the action of trypsin on horse muscle. The soluble products of hydrolysis were reduced to the dry condition (Hartley, Eagleton and Okell, 1923), and 2 p.c. solutions in normal saline made up, the reactions adjusted and the solutions sterilized.

A further quantity of purified antigen A was prepared from the same reagents as in the

previous experiment, and in the same way mixtures of purified antigen with muscle extracts and peptone solutions were made. After standing for 4 days at room temperature the mixtures were injected into guinea-pigs. As in the previous experiment, each animal received 0·2 p.c. of purified antigen together with 1·8 c.c. of muscle extract, or peptone solution, and 74 to 77 days later the antitoxin content of the serum of each animal was determined.

The results of this experiment are given in Table II, and confirm those obtained in the previous experiment. Muscle extract, whether derived from horse, ox or guinea-pig, affects adversely the activity of the purified antigen, while peptones, used in the amounts shown, exert no such deleterious action. The results of the experiments with the laboratory-made peptones (Series 18 and 19) are of special interest, and furnish additional evidence that muscle extract affects adversely the activity of the antigen. The horse-serum albumin used was a carefully purified and recrystallized specimen, and the peptic digest prepared from it contained proteoses and peptones, but no products similar

TABLE II.

A = antigen = washed floccules from a neutral diphtheria toxin-antitoxin mixture.

Series.	Injected with—	pH.	Bled after days.	Antitoxin produced : units per c.c.					
10	0·2 c.c. A + 1·8 c.c. saline	7·6	74	0·1,	0·1,	0·1,	0·1,	0·04,	0·02
11	0·2 c.c. A + 1·8 c.c. beef extract	7·4	74	0·3,	0·02,	0·02,	0·005,	0·005,	0·002
12	0·2 c.c. A + 1·8 c.c. veal extract	7·2	74	0·2,	0·04,	0·04,	0·02,	0·002,	<0·002
13	0·2 c.c. A + 1·8 c.c. horse extract	7·2	75	0·4,	0·15,	0·04,	0·01,	0·002,	<0·002
14	0·2 c.c. A + 1·8 c.c. guinea-pig extract	7·2	75	0·1,	0·02,	0·02,	0·02,	0·01,	<0·002
15	0·2 c.c. A + 1·8 c.c. Parke Davis peptone	7·4	75	0·5,	0·3,	0·3,	0·2,	0·15,	0·005
16	0·2 c.c. A + 1·8 c.c. "Difco" peptone	7·6	77	0·3,	0·15,	0·1,	0·1,	0·1,	0·01
17	0·2 c.c. A + 1·8 c.c. Witte peptone	7·4	77	0·1	0·1,	0·1,	0·1,	0·04,	0·002
18	0·2 c.c. A + 1·8 c.c. of solution of dried peptic digest of serum albumin	7·6	77	>0·5,	>0·5,	0·3,	0·1,	0·1,	0·005
19	0·2 c.c. A + 1·8 c.c. of solution of dried tryptic digest of horse muscle	7·4	77	0·1,	0·04,	0·04,	0·04,	0·02,	0·005

to those obtained by the aqueous extraction of muscle ; the substance behaved like the other peptones studied. On the other hand, the tryptic digest of horse muscle contained, in addition to the products of hydrolysis of muscle protein, all the muscle extractives, and this product, like the other four muscle extracts studied, depressed the activity of the purified antigen.

In Table III the antitoxin production in 119 guinea-pigs which received the same dose of purified antigen, A (made on different occasions from the same reagents and by the same method), is shown. The antitoxin content

TABLE III.

Group.	Injected with—	Antitoxin produced: units per c.c. serum.														
		>0·5.	0·5.	0·4.	0·3.	0·2.	0·15.	0·1.	0·08.	0·06.	0·04.	0·02.	0·01.	0·005.	0·002.	<0·002.
I.	0·2 c.c. antigen A + 1·8 c.c. normal salt solution	5	2	1	2	8	2	17	..	..	2	2	..	..	..	..
II.	0·2 c.c. antigen A + 1·8 c.c. 2 p.c. peptone in normal salt solution	2	1	..	4	4	2	12	..	..	1	..	1	2	1	..
III.	0·2 c.c. antigen A + 1·8 c.c. muscle extract	...	..	1	1	2	1	3	1	2	8	13	3	5	5	3

was determined 74 to 78 days after injection. The figures show the number of animals which produced the amount of antitoxin stated at the head of the columns. In Group I, in which the antigen was injected in normal salt solution, 37 of the 41 guinea-pigs (90 p.c.) produced 0·1 unit or more of antitoxin per c.c.; in Group II, in which the antigen was injected in 2 p.c. peptone in normal salt solution, 25 of the 30 guinea-pigs (83 p.c.) produced 0·1 unit or more of antitoxin per c.c.; while in Group III, in which the antigen was injected in muscle extract, only 8 of the 48 guinea-pigs (17 p.c.) produced 0·1 unit or more of antitoxin per c.c.

Experiment 3.

A further experiment was carried out, using a purified antigen, B, made from a different toxin and antitoxin, and the effect of larger quantities of meat extract and peptone on the activity of this second antigen was studied.

The antigen used consisted of the well-washed precipitate which separated from a slightly toxic mixture of toxin (200 c.c.: L_0 dose, 0·08 c.c.) and antitoxin (4·1 c.c.: 580 units per c.c.), the flocculés being finally emulsified in 20 c.c. of normal salt solution.

The following dilutions of the stock antigen, B, were prepared:

(1) 2·5 c.c. purified antigen B + 47·5 c.c. normal salt solution.
(2) „ „ „ „ 2 p.c. Parke Davis peptone in normal salt solution, pH 7·6.
(3) „ „ „ „ muscle extract (beef), pH 7·4.

The mixtures were allowed to stand for 4 days, and then injected into guinea-pigs. Twelve animals were used for the study of each mixture and each animal received 4 c.c. quantities, *i. e.* 0·2 c.c. antigen + 3·8 c.c. of normal salt solution, peptone solution or muscle extract. 75 to 76 days later the animals were bled and the antitoxin content of the serum determined. The results are given in Table IV. The figures show the number of guinea-pigs which produced the amount of antitoxin stated at the head of the columns.

In the earlier papers referred to it was shown that the flocculés prepared from a toxic mixture are more active antigens than those prepared from neutral or over-neutralized mixtures, and this is again illustrated in these experiments. Antigen B prepared from a toxic mixture is a more potent antigen than antigen A prepared from a neutral mixture. The results of Experiment 3 are in agreement with those obtained in earlier experiments in so far as the effect of muscle extract is concerned; antitoxin production in this group is

TABLE IV.

Series.	Injected with—	Antitoxin produced: units per c.c. of serum.										
		0·5.	0·4.	0·3.	0·25.	0·2.	0·15.	0·1.	0·04.	0·02.	0·01.	0·005
20	0·2 c.c. purified antigen B + 3·8 c.c. normal salt solution	3	2	2	2	1	2	..	..	..	..	..
21	0·2 c.c. purified antigen B + 3·8 c.c. 2 p.c. Parke Davis peptone in normal salt solution	1	..	2	..	1	..	2	2	2	..	2
22	0·2 c.c. purified antigen B + 3·8 c.c. beef muscle extract	..	..	..	2	1	..	3	4	1	..	1

much lower than in the corresponding group which received the same dose of antigen in salt solution. The results obtained with peptone solution, however, are different; in the earlier experiments the peptone exerted little or no effect on the activity of the antigen, while in Experiment 3 the effect was about the same as that obtained with muscle extract. The effect may be due to the increased quantity of peptone injected with the antigen, or it may be due to the greater susceptibility of the slightly toxic antigen B to the action of peptone, since a similar effect of peptone was observed in Experiment 4, in which the antigen used as secondary stimulus was diphtheria toxin.

B. *The Effect of Peptone Solution and Muscle Extract on Antitoxin Production in Immunized Guinea-pigs.*

Experiment 4.

Eighteen animals were chosen from a larger group of 36 animals, of which the previous history was as follows: On July 28th, 1927, each animal was injected with 0·2 c.c. of a toxin-antitoxin floccule preparation of diphtheria antigen. On October 10th and 11th a small sample of blood was withdrawn from each animal for the purpose of another experiment. On October 25th a second sample of blood was withdrawn and the antitoxin content of the serum determined. From the 36 guinea-pigs so examined, 18 were chosen, the antitoxin content of the serum of the group being from 0·5 to 0·1 unit per c.c. These 18 animals were divided into three groups of 6 which corresponded exactly as regards their antitoxin content on October 25th, the 89th day following the injection of the same dose of the same purified antigen. On November 10th each guinea-pig received by subcutaneous injection 0·05 c.c. of a diphtheria toxin, the L_0 dose of which was 0·1 c.c., in a total volume of 5 c.c. Six animals received the dose of toxin in normal salt solution, 6 in muscle extract (pH 7·4), and 6 in a 2 p.c. solution of Parke Davis peptone in normal salt solution (pH 7·4). Determinations of the antitoxin content of the serum of each guinea-pig were made on the 5th, 8th, 12th, 16th, 20th, 25th and 30th day after injection of the diphtheria toxin.

The results of the experiment are collected in Table V, and in Fig. 1 the rate and extent of the antitoxin production in the three groups of guinea-pigs is illustrated. The results show that the group of animals which received the toxin dose in normal salt solution produced far more antitoxin than those which received the same dose of the same toxin in muscle extract or peptone solution.

DISCUSSION OF RESULTS.

Since these experiments were completed in 1927, many others have been carried out in the attempt to explain the action of muscle extract and peptone solution on the antigenic activity of toxin-antitoxin floccule preparations.

TABLE V.

No. of guinea-pig.	Antitoxin before injection.	Injected with—	Antitoxins: units per c.c. after days. 5.	8.	12.	16.	20.	25.	30.
1	0·5	0·05 c.c. toxin + 4·95 c.c. normal salt solution	2·0	40	40	30	30	20	15
2	0·4		0·5	15	40	40	30	20	15
3	0·3		1·0	30	50	50	40	30	20
4	0·25		0·5	60	80	100	60	50	30
5	0·2		1·0	30	80	100	60	40	30
6	0·1		0·1	2	20	15	10	10	5
7	0·5	0·05 c.c. toxin + 4·95 c.c. extract	1·0	15	20	20	20	20	10
8	0·4		1·0	10	10	15	10	10	5
9	0·3		0·5	5	5	5	5	5	2
10	0·25		1·0	20	30	30	20	10	5
11	0·2		0·1	15	15	20	20	15	10
12	0·1		0·5	15	15	15	10	10	5
13	0·5	0·05 c.c. toxin + 4·95 c.c. peptone solution	1·0	5	10	10	5	5	2
14	0·3		0·5	5	5	5	5	5	2
15	0·3		1·0	5	10	5	5	2	2
16	0·25		2·0	40	30	40	30	20	10
17	0·2		0·5	2	5	5	5	2	2
18	0·1		1·0	10	5	5	5	5	2

The possibility that the solutions used contained nitrogenous substances of such molecular complexity as to possess antigenic function, and that those substances might engage preferentially the immunizing mechanism, was

FIGURE I

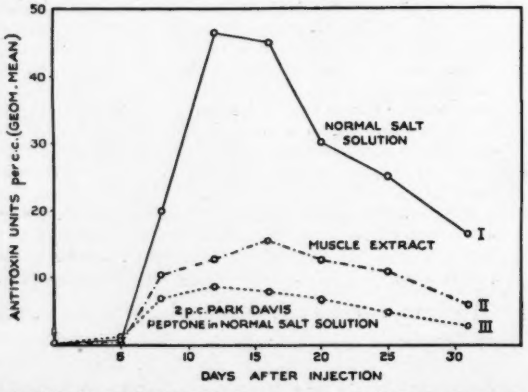

FIG. 1.—Showing antitoxin production in three groups of similarly immunized guinea-pigs in which the secondary stimulus (0·05 c.c. diphtheria toxin) was injected in 5-c.c. quantities of (1) normal salt solution; (2) muscle extract; (3) 2 p.c. Parke, Davis peptone in normal salt solution.

considered and investigated. Young virgin guinea-pigs were injected with 5 c.c. quantities of the muscle extract and peptone solution used, and the uteri tested for specific sensitization by the method of Dale and Laidlaw. The

results were uniformly negative. The possibility that muscle extract or peptone solution might exert a destructive action on diphtheria toxin (Experiment 4) was also considered and investigated. Dilutions of toxin in salt solution, muscle extract and peptone solution similar to those used in Experiment 4 were prepared and incubated at 37° C. for 6 hours ; no evidence of destruction of toxin was obtained. On the contrary, evidence was obtained (confirmed in other experiments in which freshly-prepared broth was used as diluent for the toxin) that the toxicity increased under these conditions, as found by Walbum (1923), and Schmidt and Scholz (1926).

While an explanation of the effects observed cannot yet be offered, it is considered that the results obtained in these experiments show that muscle extract and the peptones used in bacteriological work are not indifferent substances, but when injected with certain antigens at least play some part in, and under certain conditions may interfere with, the development of immunity. The results obtained with muscle extract were consistent ; when the antigen was injected in muscle extract the amount of antitoxin produced was invariably smaller than the amount produced by the same dose of antigen injected in normal salt solution, and the effect was elicited with relatively small quantities of extract. Some evidence was obtained to show that the effect produced by peptone is related to the quantity injected with the antigen.

The results recall some observations made by Glenny and Walpole (1915), who immunized two horses with diphtheria toxin purified by the method devised by Walpole (1915) and obtained antitoxins containing 800 and 1400 units per c.c. Although, as these authors point out, occasional horses gave antitoxins of higher potency, the average value of the antitoxins obtained with ordinary toxin at the time of experiment was about 700 units per c.c. In another case a horse which was failing as an antitoxin producer yielded, after injection of small doses of toxin purified by Walpole's method, antitoxin of higher unitage than it had ever produced before. These observations, though few, suggest that antitoxin production may be favoured by the use of purified toxins. Immunization by toxins purified by Walpole's method has not, however, been widely used ; the method is complicated, and it has been possible to reduce the proportion of impurities—peptone and muscle extract—to antigen in another way, viz. by the use of high potency toxins. These are now commonly and regularly obtained of a potency very many times greater than was the case in the years before the war, and it is possibly of significance that in some laboratories in which these high-grade toxins have been used antitoxins of remarkably high potency have been produced.

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THE EFFECT, ON THE DEGREE OF IMMUNITY PRODUCED, OF INJECTING THE SAME DOSE OF DIPHTHERIA FORMOL-TOXOID CONTAINED IN DIFFERENT VOLUMES OF NORMAL SALT SOLUTION.

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In some experiments recently carried out at the State Institute for Experimental Therapy, Frankfurt, the State Serum Institute, Copenhagen, and the National Institute for Medical Research, London, in which the same four diphtheria formol-toxoids were injected into guinea-pigs, and the immunity response estimated by the injection, 28 days later, of the same sample of dry diphtheria toxin, it was clearly demonstrated that the degree of immunity attained by the guinea-pigs used at Hampstead was considerably greater than that developed by the guinea-pigs used at Frankfurt and Copenhagen.

The results obtained in these comparative tests suggested that the differences observed were due, either to variation in the resistance or susceptibility of the different stocks of guinea-pigs used in the three institutes, or to small differences in the way in which the tests were carried out. Accordingly, the study of the factors which may influence the development of immunity has been continued, and in the present paper it is shown that, other conditions of experiment being the same, the degree of immunity produced by the injection of formol-toxoid is markedly influenced by the volume of fluid in which the dose of antigen is contained.

For the experiments recorded below a purified and highly concentrated diphtheria formol-toxoid, B.31, containing 850 flocculation units per c.c., was used. This had been prepared at the State Serum Institute, Copenhagen, and used for another series of comparative experiments. It had previously been shown (Hartley, 1935*a*) that it was an effective antigen. For use in the present series of experiments, a first dilution was made by adding one volume of toxoid B.31 to 84 volumes of normal salt solution, and the further dilutions of the toxoid required for the experiments were prepared from this first dilution.

The purified preparation B.31 was used in order to eliminate as far as possible the effect of the constituents of culture media on the activity of the specific diphtheria antigen (Hartley, 1935*b*).

The guinea-pigs used were all drawn from the stock maintained at Rhodes Farm, and weighed from 240 to 270 g. at the beginning of the experiment. Animals of exactly corresponding weight were used in the groups of guinea-pigs immunized, and the feeding and housing conditions were constant. No guinea-pig died during the period of immunization, and every animal increased in weight during this period.

EXPERIMENTAL.

In the first experiment 15 guinea-pigs were injected subcutaneously, each with 5 flocculation units contained in a volume of 0·5 c.c. of normal salt solution, and 15 similar guinea-pigs were injected with 5 flocculation units contained in a volume of 5 c.c. of normal salt solution. Twenty-eight days after injection the guinea-pigs were tested for immunity by application of the multiple Schick test dose method (Glenny, Hopkins and Pope, 1924), the doses of diphtheria toxin injected into each guinea-pig being 1, 2, 5, 10, 25 and 50 Schick test doses, each skin test dose being contained in a volume of 0·2 c.c. The guinea-pigs maintained for use at this Institute are all white, cream, or cream and white in colour, and consequently when the toxin doses are injected at wide intervals in the skin the reading of the reactions, made 1 and 2 days after injection, presents no difficulty.

The results of this experiment are collected in Table I.

TABLE I.

Group and average weight.	Antigen injection.	Average weight 28 days after injection.	Proportion of guinea-pigs reacting negatively to the following Schick doses of toxin, 28 days after injection.					
			1.	2.	5.	10.	25.	50.
I (259 g.)	Each of 15 guinea-pigs injected with 5 flocculation units of B.31 contained in 0·5 c.c. salt solution	393 g.	7/15	5/15	3/15	1/15	1/15	0/15
II (260 g.)	Each of 15 guinea-pigs injected with 5 flocculation units of B.31 contained in 5·0 c.c. of salt solution	405 g.	13/15	13/15	11/15	9/15	9/15	6/15

The difference in the degree of immunity produced in the two groups is striking. Whereas less than half of the guinea-pigs receiving the dose of antigen in a volume of 0·5 c.c. failed to be immunized to the Schick level, 13 of the 15 guinea-pigs which received the same dose of antigen in a volume of 5 c.c. became Schick-negative; and while only one of the animals in the first group reacted negatively to 25 Schick test doses, 9 of the second group reacted negatively to this dose of toxin, and 6 of the second group reacted negatively to 50 Schick test doses, while none of the animals of the first group gave a negative reaction to 50 Schick test doses.

Further, the total amount of toxin injected intracutaneously into each guinea-pig (93 Schick test doses) is approximately one lethal dose for guinea-pigs of 400 g. weight. Four of the guinea-pigs receiving the dose of antigen in a volume of 0·5 c.c., when subsequently tested for immunity, died from diphtheria toxæmia, while in the group which received the same dose of antigen in a volume of 5 c.c. all survived without symptoms—a further demonstration of the higher degree of immunity developed in the group of animals which received the antigen in the larger volume of salt solution.

A second experiment was carried out to investigate the effect of varying the volume in which the dose of antigen is contained when *two* injections are given subcutaneously to similar groups of guinea-pigs.

The doses injected and the intervals between the injections being the same throughout, 15 guinea-pigs received each of the 2 doses of antigen in a volume of 0·5 c.c. (Group A); 15 guinea-pigs received each of the 2 doses of antigen in a volume of 5 c.c. (Group D); 15 guinea-pigs received the first dose in a volume of 0·5 c.c., and the second dose in a volume of 5 c.c. (Group B); and 15 guinea-pigs received the first dose in a volume of 5 c.c., and the second dose in a volume of 0·5 c.c. (Group C). Nine days after the second injection each of the 60 guinea-pigs was tested for immunity by the application of the multiple Schick dose test. The results are given in Table II.

TABLE II.

Group and average weight.	First injection dose, B.31 (flocculation units).	Volume in which the first dose was contained (c.c.).	Interval between injections (days).	Second injection dose, B.31 (flocculation units).	Volume in which the second dose was contained (c.c.).	Interval between second injection and multiple Schick test (days, and average weight).	Proportion of guinea-pigs reacting negatively to the following Schick test doses of diphtheria toxin.				
							1.	2.	5.	10.	25.	50.
A (264 g.)	1	0·5	19	1	0·5	9 (402 g.)	7/15	6/15	5/15	5/15	5/15	3/15
B (263 g.)	1	0·5	19	1	5·0	9 (396 g.)	5/15	5/15	5/15	5/15	5/15	4/15
C (259 g.)	1	5·0	19	1	0·5	9 (409 g.)	14/15	14/15	14/15	13/15	10/15	8/15
D (266 g.)	1	5·0	19	1	5·0	9 (410 g.)	15/15	15/15	15/15	14/15	13/15	13/15

Considering first the results obtained in the animals of Group D and Group A, it is clear that the degree of immunity attained when the doses were given in the larger volume (5 c.c.) was much greater than that attained when they were given in the smaller volume (0·5 c.c.). In the former case (Group D) all the 15 guinea-pigs were Schick-negative 9 days after the second injection, and 13 of the 15 reacted negatively to the large dose of 50 Schick test doses of toxin, while in the latter case (Group A) only 7 out of the 15 reacted negatively to one Schick test dose and only 3 reacted negatively to 50 Schick test doses. All the animals in Group D remained well and showed no symptoms, while 4 of the 15 animals in Group A died as a result of the injection of toxin. The results are in agreement with those recorded in the preceding experiment.

The results obtained with the animals of Group B were very similar to those recorded for Group A, and suggest that when the primary stimulus is injected in a small volume of fluid, the primary response is relatively small, since, as a result of the secondary stimulus, even when this was injected in a volume of 5 c.c., only 5 of the 15 animals became immunized. In this group also 4 of the 15 animals died as a result of the injection of toxin. On the other hand, the results obtained with the animals of Group C suggest that when the primary stimulus is injected in a volume of 5 c.c., the primary response is marked, since the injection of the second dose, even when contained in a volume of 0·5 c.c., resulted in a high degree of immunity in the group; all the 15 animals in Group C survived the injection of the 93 Schick test doses of toxin.

DISCUSSION OF RESULTS.

The marked difference in the results obtained may be associated with the rate of absorption and elimination of the antigen. When the antigen is given in a volume of 0·5 c.c. all signs of swelling or thickening have disappeared in 4 to 6 hours, whereas when it is given in a volume of 5 c.c., much of the injected fluid remains unabsorbed at the end of this time; absorption is usually complete, however, 24 hours after injection. Glenny, Buttle and Stevens (1931) have shown that diphtheria formol-toxoid is rapidly eliminated, and it is possible that, when given in the larger volume, the antigen remains longer at the site of injection and in the body. This may well be the explanation, but if so, it is

rather remarkable that such a relatively short delay in absorption should make such a large difference in the final result. It is possible that other factors are brought into play. When the antigen is presented in the larger volume a greater number of antitoxin-producing cells may be engaged and stimulated; for the development of immunity it may be better to stimulate a large number of cells with a relatively small, yet adequate quantity of antigen, than to stimulate fewer cells with what may be more than an optimal dose.

The results are recorded at this stage because they may have important practical applications. In the study of the immunizing properties of diphtheria and possibly other antigens, for example, the volume in which the dose of antigen is injected may have an important bearing on the final results, and should accordingly be taken into consideration.

The facilities for extending these experiments to the active immunization of larger animals, including man, are not available to the writer; other workers, however, may be interested to discover whether the introduction of such modifications of present-day technique as are suggested by the results quoted above would be an advantage.

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NOTE ON THE EFFECT OF INJURY ON THE LEVEL OF THE PLASMA PROTEINS.

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IT has long been known that the level of fibrinogen in the blood plasma is very variable, and that acute inflammations are invariably associated with high values. Further, a deficit of fibrinogen in contrast to the other proteins can be restored in a few hours. Foster and Whipple (1921–22) showed that dead tissue and a non-specific (aseptic) inflammation are the only factors necessary to cause this increase of fibrinogen in the blood. W. and H. Lohr (1922) and Govaerts and de Harven (1928) noted a rise in the fibrinogen content of the plasma following aseptic operations. In addition W. and H. Lohr observed a reversal in the albumin : globulin ratio following aseptic operations and accidental injuries. The absolute amounts of albumin and globulin, however, are not recorded in their paper. Reinmann, Medes and Fisher

(1933) believe that the increases in globulin and fibrinogen may, in part, be due to the decomposition of leucocytes, especially of the polymorphonuclear type in the circulation or in the hæmatopoietic system.

The following observations were made with a view to assessing the time and nature of the changes in the level of the plasma proteins which result from traumatic injury and surgical intervention.

Methods Employed.

The venous blood was withdrawn without stasis into tubes containing a standard minimum amount of oxalate. Peters, Eisenmann and Bulger (1925) have found that oxalate causes a dilution of the plasma such as to reduce the total protein content by 0·3–0·4 g. per 100 ml., and recommended the use of heparin. Although heparin was not used in the present series of observations, the dilution effects would be relatively constant and should not affect the significance of the data, since the emphasis is upon the relative and not absolute changes in the level of the plasma proteins.

The fractionation and determination of the plasma proteins was performed by the composite method described by Hawk and Bergeim (1927).

Results.

Salvesen's (1926) data on sixteen normal men may be taken as a basis from which to judge the deviation found to result from injury. The maximum and minimum values found for albumin were 5·24 g. and 3·95 g. per 100 ml. plasma (mean 4·44); for globulin, including fibrinogen, 3·18 g. and 1·96 g. (mean 2·58). Peters and Van Slyke (1931) quote as extreme values for fibrinogen 0·4 g.–0·2 g. Rowe (1917) has noted that excessive protein feeding does not increase the plasma protein content above the usual normal limit, so that it is unlikely that the effects noted in Cases 1 and 3 were due to the large amounts of egg-white and sodium caseinate which they respectively consumed. Schenk and Kunstmann (1933) have found that the level and amino-acid constitution of the proteins of the blood serum may vary from individual to individual, and in the same individual during the course of the day. The main differences occurred in the albumin fraction.

The details of the cases investigated are given in the table.

Case 1 exhibits the series of changes in the plasma proteins following an injury such as the fracture of both bones of both legs. The first observation made 5 hours after the accident and before manipulation under anæsthetic, revealed a normal partition of the proteins. Subsequent analyses showed a rise in total protein due to marked increase in the globulin fraction accompanied by a slight rise in fibrinogen. The albumin moiety declined. These changes subsided about the fourth day, and then returned to their previous level about the end of the first week. At the end of 16 days a normal ratio was found. Irregular fluctuations, chiefly in the globulin fraction, were also noted in Case 2. That these fluctuations in the level of the plasma proteins may follow disturbances at the injured site is noted in Case 3. This case also exhibited a rise, then a fall in the globulin fraction. On the seventeenth day after the accident the leg was manipulated under anæsthesia (chloroform and

Case.	Time after accident or operation.	Plasma protein N g. Total.	Albumin.	Globulin.	Fibrinogen.	Plasma N.P.N. mg.	Nature of accident or operation.
1	5 hours	7·11	4·14	2·80	0·17	24	Fracture both bones of both legs by direct violence. Legs manipulated under general anæsthetic 9–10 hours after accident.
	24 „	8·05	4·54	3·15	0·36	30	
	2 days	9·45	3·18	5·98	0·29	30	
	3 „	9·30	3·22	5·78	0·30	25	
	4 „	7·71	3·54	3·97	0·20	26	
	6 „	9·49	2·50	6·61	0·38	32	
	7 „	9·49	3·13	6·36		25	
	8 „	7·97	3·24	4·50	0·23	24	
	16 „	7·78	4·86	2·74	0·18	26	
2	22 hours	6·80	3·02	3·78		23	Fracture of femur.
	2 days	8·56	2·90	5·40	0·26	26	
	4 „	6·80	3·21	3·36	0·23	27	
	6 „	8·41	2·96	5·24	0·21	26	
	9 „	7·55	3·50	3·86	0·19	24	
	11 „	7·24	3·34	4·64	0·26	26	
	14 „	7·55	3·46	3·92	0·17	26	
3	24 hours	6·98	2·64	3·71	0·63	23	Compound fracture of both bones one leg. On 17th day manipulated under general anæsthetic and 2½ hours later blood withdrawn.
	7 days	9·71	2·95	6·30	0·46	32	
	14 „	6·96	3·58	3·15	0·23	32	
	17 „	15·06	4·92	9·07	1·07	43	
	18 „	10·97	5·38	5·00	0·59	33	
	21 „	7·10	3·15	3·78	0·17	20	
4	Before operation	9·56	4·10	5·46		31	Excision of knee-joint under general anæsthetic (35 min.) to correct deformity due to old osteomyelitis in childhood; now 17½ years.
	1½ hours	9·81	4·05	5·40	0·36	20	
	2¾ „	11·42	3·79	6·82	0·81	23	
	5¼ „	11·73	3·79	7·08	0·86	24	
	24 „	13·01	2·63	9·64	0·74	24	
	2 days	10·21	3·91	6·10	0·20	33	
	3 „	10·08	3·78	6·14	0·16	30	
	6 „	10·08	3·78	6·05	0·25	26	
	14 „	6·89	4·56	2·14	0·19	26	
	22 „	6·28	3·78	2·34	0·16	26	
5	1 day	6·80	3·02	3·78		23	Effusion into knee-joint as result of fall from car. This case was of same sex, build and age as Case 6.
	2 days	8·56	2·90	5·40	0·26	26	
	4 „	6·80	3·21	3·36	0·23	27	
	6 „	8·41	2·96	5·24	0·21	26	
	9 „	7·55	3·50	3·86	0·19	24	
6	2 days	6·86	2·80	4·06		26	Effusion into knee-joint as result of fall.
	3 „	12·15	2·71	9·12	0·32	24	
	5 „	7·23	4·21	2·83	0·19	26	
	7 „	8·80	3·78	4·79	0·23	24	

ether). A very marked rise in the total protein was noted in a sample of blood withdrawn 2½ hours after this procedure. The rise was principally due to a great increase in globulin and fibrinogen fractions. This change soon subsided.

The most complete record of these fluctuations following injury is that of Case 4. It includes data collected both before and after an excision of the knee-joint of a lad of 17½ years. This operation was performed to correct a

deformity due to osteomyelitis in childhood. The pre-operation level of the total protein was high, due to an abnormal content of globulin, possibly caused by the persistence of some chronic inflammatory process. As a result of the operation there was a rapid and marked rise in globulin and fibrinogen accompanied by a slight rise in the albumin fraction. The maximum change was noted 24 hours after the operation. Subsequently both globulin and fibrinogen declined, while the albumin fraction rose. Hæmatocrit determinations did not indicate any change in blood volume to account for these marked fluctuations.

That anæsthesia and actual fracture of bone may be discounted as agents peculiar to this disturbance in the plasma proteins may be inferred from Cases 5 and 6. Each of these men, who were of similar build and age, suffered from an effusion into a knee-joint as the result of a fall. It will be noted that also in these cases there was a rise in the plasma proteins mainly due to increases in the globulin fraction. The maximum recorded changes varied in time and degree. Fuller investigation of the first 24-hour period might have revealed earlier changes.

DISCUSSION.

A satisfactory explanation of these phenomena cannot be offered, although somewhat similar changes have been observed in the level of the blood fat, following operation (Prochnow and Findeisen, 1933). In surgical shock and allied states the plasma proteins do not rise, but fall, in spite of the fact that the hæmoglobin increases (White and Erlanger, 1920). In the present series of cases shock was practically absent, and the injury was rapidly succeeded by a period characterized, in particular, by an increase in globulin and fibrinogen. This change is probably intimately related to the process of healing, involving as it does proliferation and phagocytosis. The recuperative reserves of the organism are thereby made available. Whipple and his co-workers (1935) state that in an emergency plasma protein may contribute to body protein. They found that in their dogs there was a " reserve store " of plasma-protein-building substance which might amount to 30–120 g. of potential material—twice or three times as much on the average as the dog has in its circulation. They suspect the liver and perhaps other tissues as the reserve store for this material, which may be used for plasma protein regeneration or for tissue repair. This is in general agreement with the views of Reinmann, Medes and Fisher (1934). That the plasma proteins can be utilized in the body economy appears evident from the experiments of Holman, Mahoney and Whipple (1934), which demonstrate that dogs receiving only sugar by mouth can be maintained practically in N equilibrium by suitable amounts of blood plasma given intravenously.

The recent work of McFarlane (1935) has thrown doubt on the homogeneity of the globulin fraction as partitioned by neutral salt precipitation. A variety of sera—normal and pathological, human, cow and horse—were treated with ammonium sulphate in accordance with the usual fractionation technique. The globulin precipitates were redissolved in 1 p.c. NaCl, dialysed free of SO_4 ions and examined in the ultracentrifuge. In some cases practically no

albumin was present; in others there were two sedimenting boundaries corresponding in sedimentation rate to albumin and globulin—indeed as much as 40% of this fraction was sometimes albumin. There appears to be no simple explanation of this phenomenon.

So far no attempt has been made to apply ultracentrifugal analysis to the plasma of injured persons, so that it cannot be stated that the observed increase in globulin is a true increase. Should it be found that the observed increase is due to the presence of albumin, the marked increase in albumin still demands an explanation.

It is of interest to note that although the maximal changes in the plasma proteins precede in time the phase of increased catabolism which follows injury, and which reaches a maximum within 4–11 days from the date of injury (Cuthbertson, 1930), it is probable that these phenomena are intimately related to the processes of repair and maintenance.

SUMMARY.

Trauma such as to cause the fracture of one or more of the long bones, or an effusion into a knee-joint or a surgical procedure such as the excision of a knee-joint, causes an immediate and marked disturbance in the total amount and relative proportions of the different plasma proteins as fractioned by neutral salt precipitation. The normal level is slowly regained.

The general effect is a slight fall in the albumin moiety coupled with a marked rise in the globulin fraction. Fibrinogen is often appreciably raised.

The writers wish to thank their clinical colleagues for permission to investigate these cases.

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THE RELATIONSHIP BETWEEN THE HETEROPHILE HAPTEN AND THE SPECIFIC POLYSACCHARIDE HAPTEN OF THE "SMOOTH" FORM OF *BACT. SHIGÆ.*

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IIJIMA (1923) was the first to show that a hæmolytic antibody for the red cells of the sheep and goat, having the character of a heterophile or 'Forssman' antibody, appears in the serum of rabbits immunized by intravenous injections of heat-killed suspensions of *Bact. shigæ*. He believed that 'Shiga' bacilli could adsorb the 'Forssman' hæmolytic antibody, which is obtained by immunizing the rabbit with a suspension of kidney tissue from the guinea-pig, that such a suspension—the Forssman-antigen in the strict sense—was able to fix and remove the hæmolytic antibody from 'Shiga' sera, and that an aqueous extract of Shiga's bacillus could bind complement in the presence of Forssman-antibody. Fujita (1924) confirmed the presence of a heterophile antigen in Shiga's bacillus, but he was unable to demonstrate the fixation of complement and the adsorption of the 'Forssman' antibody by a suspension of 'Shiga' bacilli. He found that the heterophile antigen in Shiga's bacillus is insoluble in alcohol and is, therefore, probably not lipoidal in nature. Kurt Meyer (1926) showed that the 'Shiga' heterophile antibody is adsorbed only by guinea-pig tissues or by a suspension of Shiga's bacillus, and not by other closely related enteric organisms nor by some unrelated bacteria. The researches of Jungeblut and Ross (1928) and Yasui (1929) on the heterophile antigen in bacteria of the paratyphoid-dysentery group largely confirmed the findings of Fujita and Meyer. Meyer (1930, 1931) found that the carbohydrate which had been isolated in crude form by fractionation of the bacterial cell substance possessed the property of neutralizing the specific hæmolysin in the same way as an extract of *Bact. shigæ*. The amount of the heterophile "antigen" in the crude polysaccharide, when measured by its ability to neutralize the 'Shiga' heterophile hæmolysin, showed a constant relation to the amount of the "species-specific carbohydrate"* contained in the preparation. The latter could be measured by using, in complement-fixation tests, an antibacterial serum prepared from *Bact. shigæ* which was free from heterophile antibody. Meyer concluded that the heterophile "antigen" and the "species-specific carbohydrate" were probably contained in the same complex molecule. He believed that a single substance of this type which

* In the present paper we have replaced the term "species-specific carbohydrate" which was used throughout the earlier publications of Meyer (1930, 1931) by the phrase "specific bacterial polysaccharide hapten"; the word "bacterial" is included to signify the function of this hapten in reacting with an antibacterial serum to form a specific precipitate and to fix complement.

possesses two immunologically distinct and reactive groups may explain the production *in vivo* of both the antibacterial and the heterophile antibody.

Working independently on different lines Morgan (1931 and unpublished work) isolated the specific bacterial polysaccharide hapten in a pure condition from the " smooth " variant of *Bact. shigæ*. The specific substance is a weakly acidic polysaccharide which contains about 1·6 p.c. nitrogen, possesses a specific rotation of $[\alpha]_D + 98°$, yields over 92 p.c. of reducing sugars on acid hydrolysis, and gives definite precipitation with the homologous antibacterial immune-serum up to a dilution of 1 in 12,000,000. The nitrogen is present as an acetylated amino group which is not attacked by nitrous acid. The acetyl group is resistant to the action of dilute alkali, and, therefore, differs in this respect from the acetyl group in the type-specific polysaccharide hapten isolated from the pneumococcus (Type I) by Avery and Goebel (1933) and by Enders and Wu (1934). When the ' Shiga ' specific polysaccharide is given intravenously in doses as small as 0·01 mg., it induces a typical and fatal anaphylactic shock in guinea-pigs that have been passively sensitized with homologous immune rabbit serum (Morgan, 1932). Repeated attempts to separate other polysaccharide substances from the specific bacterial polysaccharide hapten, $[\alpha]_D + 98°$, by the fractional precipitation of aqueous or pyridine solutions by means of alcohol, acetone or acetic acid have always resulted in the recovery of the original material, unchanged in its physical, chemical and serological properties. It would appear, therefore, that it is a chemically homogeneous substance and not a mixture of different polysaccharides ; it is hoped to publish soon a detailed account of its preparation and properties.

The experiments of Kurt Meyer are now known to have been carried out with a polysaccharide preparation which contained not more than 10 p.c. of the pure specific bacterial polysaccharide hapten as characterized above ; the remaining substance was most probably non-specific polysaccharide material. The pure substance was therefore used in fresh experiments carried out on similar lines to those on which Meyer had based his conclusions concerning the relationship between the heterophile hapten and the specific polysaccharide.

EXPERIMENTAL.

Heterophile sera were produced by immunizing rabbits with mixed suspensions of " smooth " heat-killed cultures of *Bact. shigæ*. The animals were bled before immunization, and after they had received intravenously four or five weekly doses of the suspension. The sera which produced complete lysis of a suspension of sheep red-cells, in the presence of complement, at dilutions higher than 1 in 100 were considered sufficiently potent in heterophile antibody for use.

The first experiment was made in order to determine whether the pure specific bacterial polysaccharide hapten, which is known to combine readily with ' Shiga ' antibacterial immune-body, also possesses the property of neutralizing the hæmolytic action of the ' Shiga ' heterophile antibody on sheep red-cells. For control purposes several non-specific polysaccharides were included in the experiment. The minimum amount of the specific

bacterial polysaccharide hapten that was able to neutralize one hæmolytic dose of 'Shiga' heterophile antibody has been adopted as a quantitative measure of the amount of heterophile receptor in the materials used.

The Test for Neutralization of the Hæmolytic Antibody.

Decreasing amounts of a suitable dilution of the polysaccharide preparations were mixed with one hæmolytic dose of 'Shiga' heterophile serum and allowed to stand in the ice-chest for 15 minutes. A 6 p.c. suspension of sheep red cells (0·25 c.c.) and 0·25 c.c. of a 1 in 10 dilution of fresh guinea-pig serum were then added, and the mixtures, in a total volume of 1·25 c.c., were incubated at 37° until the serum control tube, which contained no polysaccharide, showed complete hæmolysis.

The results of the experiment are given in Table I. The preparation A was a sample of the pure specific bacterial polysaccharide hapten, $[\alpha]_D + 98°$. Preparation B was a specimen of the pure specific polysaccharide which had been converted into its benzoyl derivative and regenerated by cautious alkaline hydrolysis in aqueous acetone solution. Specimen C was a preparation of the

TABLE I.—*Fixation of Complement by 'Shiga' Heterophile Antibody and Various Polysaccharide Preparations.*

		HÆMOLYSIN-INHIBITION TEST.					
		Degree of hæmolysis of sheep-cells.					
Polysaccharide preparation.	Dilution of polysaccharide used. 1 in—	Volume of polysaccharide solution used.					
		0·50 c.c.	0·25 c.c.	0·10 c.c.	0·05 c.c.	0·025 c.c.	0·01 c.c.
A	5×10^6	0	tr	i	ac	c	c
B	,,	0	tr	i	ac	c	c
C	,,	tr	tr	i	ac	c	c
D	,,	0	tr	i	ac	c	c
E	10^4	0	tr	i	ac	c	c
F	,,	0	tr	i	ac	c	c
G	,,	0	tr	i	ac	c	c
H	10^3	0	tr	i	ac	c	c
J	,,	0	tr	i	ac	c	c
K	,,	c	c	c	c	..	..
L	,,	tr	i	ac	c	c	..
M	10^4	..	i	c	c	c	..
N	,,	..	c	c	c	c	..
O	,,	..	c	c	c	c	..

0, no hæmolysis; tr, trace of hæmolysis; i, incomplete hæmolysis; ac, almost complete hæmolysis; c, complete hæmolysis.

specific polysaccharide which contained 10–20 p.c. of non-specific polysaccharides; and preparation D was a sample of the specific polysaccharide which had been regenerated from the acetyl derivative of preparation A. Two specimens of polysaccharide material (E and F) that had been obtained from a "rough" culture of *Bact. shigæ* were included in the tests, and also three non-specific polysaccharides (G, H and J) that had been released from their association with the specific polysaccharide A during the course of its isolation and purification. The polysaccharide substance J had been identified as glycogen; the preparation G possessed the serological property of forming

a specific precipitate with anti-agar serum up to a dilution of 1 : 10,000,000. Specimens K and L were samples of polymannose and polygalactose that had been isolated by Clutterbuck *et al.* (1934) from the metabolic products of *Penicillium charlesii*. The heterologous bacterial polysaccharides that were tested included the following : The acetylated (M) and the de-acetylated (N) specific polysaccharide haptens of the pneumococcus (Type I), and the specific polysaccharide (O) of the pneumococcus (Type II). Polysaccharide material isolated from *Bact. flexneri* (Y), tubercle bacilli of human origin, *Bact. suipestifer*, *Bact. paratyphosus B* (Newport), *Bact. enteritidis* and *Br. abortus* were also examined, but the results obtained with these substances have not been included in the table.

The results show that 0·5 c.c. of a 1 in 5,000,000 dilution of the purest polysaccharide (preparation A) completely inhibited the lytic action on sheep red-cells of one hæmolytic dose of the ' Shiga ' heterophile antibody. The ability of preparations A, B and D to neutralize the heterophile antibody appeared to be equal, whereas preparation C, which contained some non-specific polysaccharide material, was less active. The polysaccharide substances (E, F, G, H and J) showed less than 1 p.c. of the activity of the pure polysaccharide, A ; this activity was almost certainly due to traces of the specific bacterial polysaccharide hapten in the preparations. The other polysaccharides tested caused only a partial neutralization of the hæmolytic action of the heterophile antibody, even when they were present in a concentration amounting to 500 times that of the specific substance, A.

The next experiment was carried out in order to ascertain the combining capacities of the polysaccharide preparations for the ' Shiga ' antibacterial immune-body. The method consisted in estimating the least quantity of each of the polysaccharides that would fix one hæmolytic dose of complement in the presence of an optimal quantity of a ' Shiga ' antibacterial immune-serum which was free from heterophile antibody. The results obtained give a quantitative measure of the specific receptors that are present in the polysaccharide preparations, and that are capable of combining with antibacterial immune-body.

Complement-fixation Test.

Decreasing amounts of an appropriate dilution of the polysaccharide preparations were mixed with 0·2 c.c. of a 1 in 10 dilution of anti-bacterial ' Shiga ' serum, which was free from heterophile antibody, and 0·25 c.c. of a 1 in 20 dilution of fresh guinea-pig serum. After the mixtures had been kept at 0° for 45 minutes, and 37° for 15 minutes, 0·25 c.c. of a 6 p.c. suspension of sheep red-cells that had been sensitized with an appropriate immune-serum from the rabbit was added to each tube. The mixtures, in a total volume of 1·25 c.c., were incubated at 37° until the serum control tube, which contained no polysaccharide, and the polysaccharide control tube, which contained no serum, showed complete hæmolysis.

The results of the experiments are given in Table II, where it is seen that the pure specific bacterial polysaccharide preparations A, B, and D were the most effective in fixing complement. The " rough " ' Shiga ' polysaccharides E and F and the non-specific polysaccharides G, H and J were about 100 times less active in this respect than the preparations A, B and D. The heterologous bacterial polysaccharides M, N and O showed no activity. The results obtained

by means of the precipitation technique are also given in Table II ; they are in agreement with those given by the complement-fixation experiments.

A study of the data in Tables I and II shows that in all the preparations that have been examined the amount of the heterophile receptors present bears a constant relation to the amount of specific receptors that will combine with the antibacterial immune-body. Furthermore, the minimum quantity of the polysaccharide preparation necessary to neutralize one hæmolytic dose of 'Shiga' heterophile serum was found to be about five times less than the minimum amount necessary to fix one hæmolytic dose of complement in the presence of an optimal quantity of antibacterial immune-body.

TABLE II.—*Reactions between 'Shiga' Antibacterial Serum and Various Polysaccharide Preparations.*

COMPLEMENT-FIXATION TEST.

Polysaccharide preparation.	Dilution of polysaccharide used. 1 in—	Degree of hæmolysis of sheep-cells. Volume of polysaccharide solution used. 0·50 c.c.	0·25 c.c.	0·10 c.c.	0·05 c.c.	0·025 c.c.	0·01 c.c.
A	10^6	0	tr	i	ac	c	c
B	„	0	tr	i	ac	c	c
C	„	tr	i	ac	c	c	c
D	„	0	tr	ac	ac	c	c
E	10^4	0	0	tr	i	ac	c
F	„	0	0	tr	i	ac	c
G	„	0	0	tr	i	ac	c
H	10^3	0	tr	i	ac	c	..
J	„	0	tr	i	ac	c	..
K	„	c	c	c	c	c	..
L	„	tr	i	ac	c	c	..

PRECIPITATION TEST.

Polysaccharide preparation.	Dilution of polysaccharide giving precipitation. 1 in— 1×10^5.	1×10^6.	2×10^6.	4×10^6.	6×10^6.	8×10^6.	10×10^6.	12×10^6.	Control.
A	++++	++	++	+	+	+	+	+	0
B	++++	++	++	+	+	+	+	+	..
C	++++	+	+	+	+	+	±	0	..
D	++++	++	++	+	+	+	+	+	..
E	+	0	0	0	—	—	—	—	..
F	+	0	0	0	—	—	—	—	..

These results indicate that the specific receptors for the heterophile antibody and for the antibacterial immune-substance are present in a single molecule of the specific bacterial polysaccharide hapten derived from the "smooth" form of Shiga's bacillus.

It is not possible to make a direct comparison between the results given in Tables I and II and those obtained by Meyer (1930, 1931), because the combining power of one hæmolytic dose of heterophile serum for a particular polysaccharide preparation varies with the specimen of the heterophile serum employed. We have found that the higher the hæmolytic titre of the heterophile

TABLE III.—*Showing that Different Quantities of the Specific Bacterial Polysaccharide are Required for the Neutralization of the Hæmolytic Action of One Hæmolytic Dose of Three Preparations of 'Shiga' Heterophile Serum.*

HÆMOLYSIN-NEUTRALIZATION TEST.

Serum No.	Volume of a 1 : 10 dilution of serum containing one hæmolytic dose (c.c.).	Degree of hæmolysis of sheep-cells. Volume of 1 in 10^6 dilution of polysaccharide solution used.					
		0·50 c.c.	0·25 c.c.	0·10 c.c.	0·05 c.c.	0·025 c.c.	0·01 c.c.
2	0·05	0	0	0	tr	ac	c
3	0·20	0	0	tr	i	ac	c
1	0·30	0	tr	i	ac	c	..

serum, the less of the polysaccharide hapten is necessary for the neutralization of one hæmolytic dose of the serum. Table III shows that in order to neutralize one hæmolytic dose of heterophile serum No. 1, about five times as much polysaccharide hapten was required as was necessary for the neutralization of one hæmolytic dose of serum No. 2. Similarly two and a half times as much polysaccharide was required to neutralize one hæmolytic dose of heterophile serum No. 3 as was used to neutralize one hæmolytic dose of serum No. 2. The explanation of this result is probably as follows: The 'Shiga' heterophile immune-sera contain, in addition to the heterophile antibody, a certain proportion of antibacterial immune-body (agglutinin and precipitin) which is also able to combine with the polysaccharide and which varies in amount in different sera. Since, however, the two antibodies react with different receptors in the same polysaccharide molecule, it seems probable that the reaction of the antibacterial immune substance with the corresponding receptors in the polysaccharide molecule partially prevents the heterophile receptors from combining with the molecules of the heterophile antibody, owing to the masking or protective influence exerted by the spacial disposition of the receptors that have combined with the antibacterial immune-body. Examples of reactions that normally take place between two chemical groups but which become retarded or completely inhibited by the presence of a neighbouring group are well known in organic chemistry; the effect is generally attributed to steric hindrance. This explanation of the experimental results shown in Table III receives support from details of an experiment given in Table IV. It

TABLE IV.—*Showing the Diminution in the Capacity of the Specific Bacterial Polysaccharide Hapten to Combine with Heterophile Antibody after it has been Treated with* 0·2 *c.c. of a* 1 : 100 *Dilution of a Pure Antibacterial Immune Serum.*

HÆMOLYSIN-NEUTRALIZATION TEST.

Polysaccharide.	Degree of hæmolysis of sheep-cells. Volume of 1 in 5×10^6 dilution of polysaccharide solution used.					
	0·50 c.c.	0·25 c.c.	0·10 c.c.	0·05 c.c.	0·02 c.c.	0·01 c.c.
Not treated with antibacterial serum	0	0	tr	i	ac	c
Treated with antibacterial serum	i	ac	ac	ac	ac	ac

appears that after the polysaccharide hapten has been treated with antibacterial immune-body under such conditions that visible precipitation does not take place, its capacity to neutralize the hæmolytic action of the heterophile antibody is greatly diminished. Treatment of the polysaccharide with normal serum under the same conditions causes no appreciable diminution in its capacity to combine with the heterophile antibody.

It has been possible, however, to determine the combining power of the original crude preparation of the specific carbohydrate used by Meyer, and of a sample of the pure specific bacterial polysaccharide hapten with the 'Shiga' heterophile and antibacterial immune-sera that have been used for most of the experiments described in this paper. The results showed that Meyer's original preparation of the polysaccharide was about ten times less active than the purest material (preparation A) in its capacity both to neutralize the hæmolytic action of the heterophile antibody and to combine with antibacterial immune substance. However, the ratio of the minimum amount of Meyer's polysaccharide necessary to neutralize one hæmolytic dose of 'Shiga' heterophile serum to the minimum amount of this preparation required for the fixation of one hæmolytic dose of complement was the same as the corresponding ratio determined for the pure polysaccharide A. The ratio of the combining powers of the two antibodies was, therefore, the same for crude and purified preparations of the specific polysaccharide hapten derived from the "smooth" *Bact. shigæ*.

THE EFFECT PRODUCED BY ACID AND ALKALI ON THE SEROLOGICAL PROPERTIES OF THE SPECIFIC POLYSACCHARIDE HAPTEN.

In view of the relationship which, we suggest, exists between the receptors in the specific polysaccharide of Shiga's bacillus for heterophile antibody and for antibacterial immune-body, it seemed of interest to determine whether the action of dilute acid and alkali on the pure polysaccharide material would enable us to distinguish between the two types of specific receptors that are present in the polysaccharide molecule. Some preliminary experiments carried out by Meyer (1930, 1931) with a crude preparation of specific polysaccharide made it appear that the capacity of the material to combine with antibacterial immune-substance and with 'Shiga' heterophile antibody was rapidly destroyed by the action of 0·1 *N* HCl at 100°, but that similar treatment with *N* NaOH produced no appreciable inactivation. Landsteiner and Levine (1932), however, observed that a diminution in binding capacity followed the treatment of crude aqueous extracts of *Bact. shigæ* with *N* alkali at 100° C. for 30 minutes. In view of this conflicting evidence it was desirable that the rate of destruction of both serological properties should be determined in parallel on a specimen of the pure specific bacterial polysaccharide hapten.

A sample of this pure substance was dissolved in ice-cold 0·02 *N* H_2SO_4 and yielded a solution which contained 10 mg. of the substance per c.c. Amounts of approximately 2 c.c. were filled into small glass ampoules, which were sealed and heated at 100° C. for varying lengths of time. One c.c. of each solution was then measured exactly, neutralized with 1 c.c. of 0·02 *N* NaOH, and made up to 10 c.c. with saline. The ability of the polysaccharide: (1) to

inhibit hæmolysis of sheep red-cells by the 'Shiga' heterophile serum, (2) to fix complement, and (3) to precipitate with a 'Shiga' antibacterial serum was then ascertained for the various samples. The results are shown in Table V, where it will be noted that a parallel destruction of both of the specific serological properties of the polysaccharide preparation occurred. The values obtained in the complement-fixation test, after the polysaccharide had been heated at 100° C. for a period longer than an hour, were irregular and they have not been included in the table. The destruction of the capacity of the polysaccharide to react and precipitate with antibacterial immune-body is clearly seen from the results of the precipitation tests.

TABLE V.—*The Effect Produced by 0·02 N Sulphuric Acid at 100° C. on the Specific Polysaccharide Hapten.*

HÆMOLYSIN-NEUTRALIZATION TEST.

Time of heating (minutes).	Dilution of polysaccharide used. 1 in—	Degree of hæmolysis of sheep-cells. Volume of polysaccharide solution used. 0·50 c.c.	0·25 c.c.	0·10 c.c.	0·05 c.c.	0·025 c.c.	0·01 c.c.
0	5×10^6	..	0	0	tr ?	i	ac
15	„	..	0	0	tr	i	ac
45	„	0	0	tr	i	ac	c
60	„	0	tr	i	ac	c	c
120	10^6	0	0	tr	i	ac	c
180	10^5	0	0	tr ?	i	ac	c
300	10^4	0	0	tr	i	ac	c

COMPLEMENT-FIXATION TEST.

0	10^6	0	0	tr ?	i	ac	c
15	„	0	0	tr.	i	ac	c
45	„	0	tr	i	ac	c	c
60	„	tr	i	ac	c	c	c

PRECIPITATION TEST.

Time of heating (minutes).	Dilution of polysaccharide giving precipitation. 1 in— 1×10^4.	5×10^4.	1×10^5.	5×10^5.	1×10^6.	2×10^6.	4×10^6.	6×10^6.	8×10^6.	10×10^6.	12×10^6.
0	—	—	++++	+++	++	++	+	+	+	+	+
15	—	—	++++	+++	++	++	+	+	+	±	±
45	—	—	+++	++	+	+	+	+	±	0	0
60	—	—	+++	++	+	+	+	±	0	0	0
120	++	+++	+++	+	+	+	±	0	0	0	
180	+	++	+	-	0	0	0	0	0	0	
300	tr ?	tr ?	0	0	0	0	0	0	0	0	0

Similar experiments were made by employing 0·1 *N* and 0·02 *N* NaOH, and the results of one experiment with the weaker alkali are shown in Table VI. It is evident that the combining power of the polysaccharide for the two types of antibody is much less readily destroyed by alkali than by acid, and that any loss of specific activity which occurs during the alkaline treatment affects to the same degree the power of the polysaccharide to inhibit the hæmolytic action of the heterophile antibody on sheep red-cells and its ability to combine or precipitate with an antibacterial *Bact. shigæ* serum.

TABLE VI.—*The Effect Produced by 0·02 N Sodium Hydroxide at 100° C. on the Specific Polysaccharide Hapten.*

HÆMOLYSIN-NEUTRALIZATION TEST.

Time of heating.	Dilution of polysaccharide used. 1 in—	Degree of hæmolysis of sheep-cells. Volume of polysaccharide solution used. 0·50 c.c.	0·25 c.c.	0·10 c.c.	0·05 c.c.	0·025 c.c.	0·01 c.c.
0 mins.	5×10^6	..	0	0	tr	i	ac
20 ,,	,,	..	0	0	tr	i	ac
45 ,,	,,	..	0	0	tr	i	ac
3 hours	,,	..	0	tr ?	tr	ac	c
10 ,,	,,	..	0	tr	i	ac	c
20 ,,	,,	0	tr ?	tr	i	ac	c
30 ,,	,,	0	tr ?	i	ac	ac	c

COMPLEMENT-FIXATION TEST.

0 mins.	10^6	0	0	0	tr ?	i	ac
20 ,,	,,	0	0	0	tr	i	ac
45 ,,	,,	0	0	tr ?	i	ac	c
3 hours	,,	0	0	tr	i	ac	c
10 ,,	,,	0	0	i	ac	c	c
20 ,,	,,	0	tr	i	c	c	..
30 ,,	,,	0	tr	ac	c	c	..

PRECIPITATION TEST.

Time.	Dilution of polysaccharide giving precipitation. 1 in— 1×10^5.	5×10^5.	1×10^6.	2×10^6.	4×10^6.	6×10^6.	8×10^6.	10×10^6.	12×10^6.	Control.
0 mins.	++++	+++	++	++	+	+	+	+	+	0
20 ,,	++++	+++	++	++	+	+	+	+	+	..
45 ,,	++++	+++	++	++	+	+	+	+	±	..
3 hours	++++	+++	++	++	+	+	+	+	±	..
10 ,,	++++	++	++	+	+	+	+	±	0	..
20 ,,	++++	++	+	+	+	+	±	±	0	..
30 ,,	++++	++	+	+	+	±	±	0	0	..

The experiments that have been described in this section were designed in part to test whether a polysaccharide preparation which possesses only one type of receptor may be derived by chemical methods from the specific bacterial polysaccharide hapten which contains two serologically distinct types of specific receptors, but they have proved to be unsuccessful. Further work in this direction is being carried out.

THE ACTION OF A SPECIES OF *MYXOCOCCUS* ON THE SPECIFIC BACTERIAL POLYSACCHARIDE HAPTEN.

Morgan and Thaysen (1933) isolated from decaying vegetable debris a species of myxococcus which rapidly decomposes certain specific bacterial polysaccharides, including the polysaccharide hapten of Shiga's bacillus. Further (unpublished) work has shown that the specific polysaccharides of *Bact. suipestifer*, *Bact. paratyphosus B* (Newport) and *Br. abortus* (*cf.* Favilli and Biancalani, 1934) are also rapidly attacked, and their power to form a

specific precipitate with the homologous immune-serum destroyed. Furthermore, Landsteiner (1935) has recently reported the decomposition of the Group A specific substance that he has isolated from horse saliva by using the same strain of the myxococcus.

The ease with which the myxococcus destroyed the capacity of the different specific polysaccharides to form a specific precipitate with their homologous immune-serum, led us to suppose that by the action of this organism it might be possible to inactivate the receptors in the pure polysaccharide of *Bact. shigæ* that bind antibacterial immune-substance, while leaving intact the receptors for the heterophile antibody.

The action of the micro-organism on the specific polysaccharide of Shiga's bacillus was tested in the following manner: An actively growing culture of the myxococcus (0·5 c.c.) was implanted in 20 c.c. of a synthetic mineral medium which contained 5 p.c. of a 10 p.c. aqueous extract of rabbit fæces, together with 0·01 p.c. of the pure specific polysaccharide. Samples of the solution were removed at intervals and examined for the presence of: (1) specific receptors for the antibacterial immune-body by the complement-fixation and the precipitation technique, and (2) receptors for the 'Shiga' heterophile antibody by the hæmolysin-neutralization test. The results are given in Table VII and they show that there is a progressive and parallel diminution in the two serological properties examined. The myxococcus had not, therefore, destroyed the capacity of the polysaccharide complex to react

TABLE VII.—*The Decomposition of the Specific Polysaccharide Hapten by a Species of Myxococcus.*

HÆMOLYSIN-NEUTRALIZATION TEST.

Time (hours).	Dilution of polysaccharide used. 1 in—	Degree of hæmolysis of sheep-cells. Volume of polysaccharide solution used. 0·50 c.c.	0·25 c.c.	0·10 c.c.	0·05 c.c.	0·025 c.c.	0·01 c.c.
0	10^6	..	..	0	0	tr ?	i
12	„	0	0	0	tr	i	ac
24	„	0	tr	i	i	ac	c
36	10^5	0	0	tr	i	i	ac
48	10^4	0	0	tr	i	ac	c

COMPLEMENT-FIXATION TEST.

0	10^6	..	0	0	tr ?	tr	ac
12	„	0	tr ?	i	c	c	..
24	10^5	0	0	tr	i	c	..
36	10^4	tr	i	c	c	..	..

PRECIPITATION TEST.

Time (hours).	Dilution of polysaccharide giving precipitation. 1 in: 1×10^4.	5×10^4.	1×10^5.	5×10^5.	1×10^6.	2×10^6.	4×10^6.	6×10^6.	8×10^6.	10×10^6.	12×10^6.	Control.
0	+	++	++++	+++	++	++	++	+	+	+	+	0
12	+	++	++++	++	++	+	+	±	±	0	0	..
24	++	++	+	+	+	±	±	0	0	0	0	..
36	+	+	+	+	±	0	0	0	0	—	—	..
48	±	±	0	0	0	0	—	—	—	—	—	..

with ordinary 'Shiga' antibacterial serum, while leaving unimpaired the property of combining with 'Shiga' heterophile antibody. This result provides additional evidence that in the polysaccharide preparation at all stages of its decomposition there is a constant relation between the amount of heterophile receptors on the one hand, and receptors that specifically combine with antibacterial immune-body on the other hand.

DISCUSSION.

The pure specific bacterial polysaccharide hapten that has been isolated from the "smooth" variant of *Bact. shigæ* possesses the property of neutralizing the hæmolytic action of 'Shiga' heterophile antibody on sheep red-cells, in addition to its well-known property of combining with the homologous antibacterial immune-substance as indicated in complement-fixation and precipitation tests. The examination of several crude and purified specimens of specific polysaccharide obtained from Shiga's bacillus has shown that the amount of the heterophile receptors that is present in the preparations bears a constant relation to the amount of the receptors which react with the homologous antibacterial immune-substance. This relation remains unchanged during the inactivation of the polysaccharide by treatment with acid or alkali or by the action of the myxococcus. The property of neutralizing the two distinct types of antibody appears to be specific in nature, because the examination of polysaccharide material isolated from a "rough" variant of Shiga's bacillus, various non-specific polysaccharides and several heterologous bacterial polysaccharides has failed to reveal any comparable capacity on the part of these substances to combine with either of the two antibodies in question.

On the assumption that the purified specific bacterial polysaccharide hapten, which possesses a specific rotation of $[\alpha]_D + 98°$ and a nitrogen content of 1·6 p.c., is a chemically homogeneous substance, the observations that have been described point to the specific receptors for the heterophile antibody and for the antibacterial immune-substance as existing in a single polysaccharide molecule and not as separate entities in two different polysaccharide substances. The partial inactivation of the capacity of the specific bacterial polysaccharide hapten to combine with the 'Shiga' heterophile antibody after it has been treated with 'Shiga' antibacterial immune-serum furnishes further evidence in support of this view.

More than twenty years ago Wells and Osborne (1913) produced experimental evidence which indicated that the two proteins gliadin and hordein contain at least two immunologically reactive groups, one of which is common to both proteins. This work led them to formulate the theory that a single antigenic molecule may contain two or more chemical groups, the relative activity of which determines the specific character of the antigen. Whether separate and immunologically distinct antibodies can be produced in correspondence with the different determinant groups within an antigenic molecule has, however, remained an open question. This point has recently been investigated by Hooker and Boyd (1933, 1934), who used both synthetic and natural protein antigens. These workers have produced evidence for the belief that when a single protein which contains tyrosine and histidine in its

molecule is combined with diazotized arsanilic acid, it is capable of inducing the formation of two different antibodies which possess a close relationship to the tyrosine-diazo-arsanilic acid group and the histidine-diazo-arsanilic acid group respectively. More recently, they have reported that when rabbits are immunized with carefully purified duck ovalbumin and hen ovalbumin, the resulting antibodies react at first only with the homologous antigen, but that more prolonged immunization gives rise to immune-sera which react with both proteins. The heterologous antibody can be removed by absorption with the heterologous antigen thus leaving intact the homologous antibody, and for this reason Hooker and Boyd consider that the facts harmonize with the conception that determinant groups of diverse specificity, each of which may give rise to its own specific antibody, may exist in one molecule of a pure substance. Further, the results of the investigations of Avery, Goebel and Babers (1932) and of Goebel, Babers and Avery (1934) on the specificity of the antibodies formed by certain synthetic carbohydrate-protein antigens, render it probable that a single antigen of this kind may give rise to two different antibodies, each of which is specifically related to a determinant group in the antigenic complex. It is possible to explain the differences in the serological specificity exhibited by the acetylated and the deacetylated polysaccharides of the pneumococcus (Type I) on the assumption that two distinct antibodies —a precipitin for the reactive acetyl polysaccharide hapten and a precipitin for the deacetylated substance—are produced in response to the stimulus of the type-specific antigen of the organism. The experimental results described in the present paper also support the view that a chemically homogeneous bacterial polysaccharide hapten may contain two determinant groups of diverse specificity, and that such a polysaccharide, as it exists in its unchanged antigenic condition within the living organism, may induce the formation of two antibodies, each of which is specifically related to the corresponding determinant or receptor group in the polysaccharide molecule. In order to obtain further confirmatory evidence we propose to render the pure specific bacterial polysaccharide hapten of Shiga's bacillus antigenic by combining it with protein, and to examine the immune-serum produced in rabbits by means of this antigen for the presence of heterophile antibody and antibacterial immune-substance.

SUMMARY.

(1) The pure specific bacterial polysaccharide hapten of the "smooth" form of *Bact. shigæ* which readily combines with the homologous antibacterial immune-substance, is also able to neutralize specifically the hæmolytic action of 'Shiga' heterophile antibody on the red-cells of the sheep in the presence of complement.

(2) The relation between the power of the specific bacterial polysaccharide hapten to neutralize the heterophile antibody and to give complement-fixation with antibacterial immune-serum is the same for crude or purified preparations and it remains unchanged during the destruction of these specific serological properties by the action of acid, alkali or a species of myxococcus. The constancy of this relation when considered in conjunction with the fact

that treatment of the polysaccharide with anti-bacterial immune-body greatly reduces its capacity to react with heterophile antibody, furnishes strong evidence for believing that the heterophile receptors and the specific bacterial receptors co-exist in a single homogeneous polysaccharide molecule.

(3) It has not been possible by the action of dilute acid or alkali to distinguish between the two serologically different types of receptors that are present in the specific bacterial polysaccharide hapten. Attempts to prepare a polysaccharide substance containing only heterophile receptors by submitting the specific bacterial polysaccharide hapten to the enzymic action of a species of myxococcus were unsuccessful, owing to inactivation of both types of receptors at the same rate.

(4) The fact that the chemically homogeneous specific polysaccharide hapten is able to form a specific combination with two entirely different types of antibody leads to the conjecture that a pure bacterial polysaccharide hapten may contain in each of its molecules two kinds of determinant receptors of diverse specificity.

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THE APPLICATION OF SPECIFIC LÆVULOSE DETERMINATIONS TO THE LÆVULOSE TOLERANCE TEST.

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In the last few years differences of opinion have arisen as to whether the lævulose tolerance test is of value in the diagnosis of hepatic disorders. On the whole accumulated experience has tended to show that it has some value, but is of limited application in that (1) it is by no means a delicate test, so that a normal response cannot be taken to indicate a normally functioning organ; (2) many types of response are very difficult to interpret; (3) the results are vitiated in the presence of abnormal glucose metabolism (*e. g.* diabetes).

The routine test consisting in the determination of blood-sugar for a limited period following the ingestion of from 40 to 100 g. of lævulose does not involve the actual estimation of the amount of lævulose in the blood. It seemed likely that a more satisfactory test might be established by substituting the estimation of the actual lævulose for the usual blood-sugar determination. By that method it was thought the test might be made much more sensitive, and further, since only one variable was to be taken into account, the results might be more easily interpreted. Hitherto, the difficulty of making specific lævulose determinations the fundamental principle of the test has been the absence of really satisfactory methods for the actual determination of lævulose in the presence of glucose in the blood.

Recently two simple and fairly rapid methods have been devised from this laboratory (Scott, 1935; Patterson, 1935), and the present paper is concerned with the application of the method of the former to the lævulose tolerance test for liver function. A small amount of comparative work using both tests demonstrated that they give precisely similar results.

NORMAL CASES.

The first step was to determine the normal response, using venous blood and estimating the lævulose content 1 hour and 2 hours after the ingestion of 50 g. of pure lævulose (Merck, or British Drug Houses Ltd., glucose-free). Table I records some typical results, and compares the actual lævulose content with the corresponding variation in blood-sugar values as estimated by MacLean's method (1919).

It will be seen that the range of figures obtained in normal subjects for lævulose is very narrow, being 8 to 11 mg. per 100 c.c. at the end of the 1-hour stage, and 4 to 6 mg. per 100 c.c. at the end of 2 hours. It is shown, too, that

TABLE I.—*Lævulose Test on Normal Persons.*

Series No.	Type of case.	New lævulose method. Actual lævulose. (Mg. per 100 c.c.)	Ordinary lævulose method. Total reduction (MacLean). (Mg. per 100 c.c.)
1	Normal	BL. 0	53
		½ hr. 10	70
		1 ,, 10	70
		1½ hrs. 7	85
		2 ,, 4	76
		3 ,, 1	69
2	,,	BL. 0	85
		½ hr. 8	88
		1 ,, 9	86
		1½ hrs. 8	86
		2 ,, 6	87
3	,,	BL. 0	100
		½ hr. 8	104
		1 ,, 11	102
		1½ hrs. 8	100
		2 ,, 5	98
4	,,	BL. 0	105
		½ hr. 10	109
		1 ,, 11	106
		1½ hrs. 8	106
		2 ,, 5	106
5	,,	BL. 0	97
		½ hr. 9	97
		1 ,, 7	99
		1½ hrs. 5	96
		2 ,, 3	97
6	,,	BL. 0	93
		½ hr. 10	95
		1 ,, 10	95
		1½ hrs. 8	94
		2 ,, 4	94

Blood bilirubin less than 0·5 unit per 100 c.c.
,, bile salts ,, 5·0 mg. ,,

In the above cases the urine urobilinogen varied from 0·34 to 0·73 units per 100 c.c., and the fæces content from 500 to 700 units per 100 g. dry fæces.

the blood-sugar variation is by no means within such precise limits, and consequently the normal response is much less readily defined on a blood-sugar basis. Though it was intended to work upon venous blood standards, it seemed of interest to ascertain in one normal case the correspondence between the venous and capillary blood figures. Actually very close agreement was shown between the lævulose contents of both types of blood, while the blood-sugar values displayed a much greater divergence (Table II).

TABLE II.—*Comparison of Venous and Capillary Blood after Lævulose.*

Case—Normal subject.	Venous.		Capillary.	
	Actual estimated lævulose. (Mg. per 100 c.c.)	Total reduction (MacLean's method). (Mg. per 100 c.c.)	Actual estimated lævulose. (Mg. per 100 c.c.)	Total reduction (MacLean's method). (Mg. per 100 c.c.)
Before lævulose	0	102	0	105
1 hr. after	8	102	9	121
2 hrs. after	5	97	3	92

PATHOLOGICAL CASES.

A series of pathological cases have been carefully examined for comparison with normal subjects. For the most part the cases selected are those of liver disease associated with jaundice, where the fact of some degree of liver damage may be considered as reasonably well established. Moreover, these latter cases have been fully investigated from a biochemical standpoint, and the results of this form of investigation are given alongside the lævulose response for the sake of completeness, and also to define as clearly as possible the type of case under consideration (Table III).

It is clear from the data compiled in Table III that all the well-defined cases of liver disease have lævulose values above 10 mg. per 100 c.c. at the 1-hr. stage, and above 8 mg. per 100 c.c. at the end of 2 hrs.—figures quite definitely outside the normal range. The conclusion is drawn that the lævulose tolerance performed under the conditions adopted here admits of the simple interpretation that if figures higher than those mentioned above are obtained, a deficiency of liver function is indicated. Of the two values the latter one at 2 hrs. is the more important, and it is suggested that the lævulose response might be quite accurately ascertained on the basis of this value only.

The Complication of Diabetes.

An extremely interesting case diagnosed as subacute yellow atrophy complicated by diabetes became available for investigation, which made it possible to ascertain not only the influence of the liver disease, but also the extent to which the lævulose values were affected by the complication of diabetes, since the liver condition subsequently improved to such extent that the intense jaundice originally present entirely disappeared. Opportunity was also taken to compare the venous with the capillary blood response in this case for the purpose of relating it to the normal values recorded in Table II. The data obtained on this case is separately recorded in Table IV.

In contrast to its effect on the total reducing sugar, diabetes has relatively little influence upon the lævulose figures, affecting them at the maximum to the extent of only 2 mg. per 100 c.c. Again a close agreement is found between the venous and capillary blood, there being, if anything, a tendency for the capillary blood to reach a slightly higher level at the hour stage.

Other types of disease have been investigated by the present technique, for example, cases of hæmolytic and pernicious anæmia, and two skin cases,

TABLE III.—*Lævulose Test*

The analytical methods employed were the following: Blood-sugar, MacLean (1919); Urobilinogen,
Normal values: Urobilinogen in urine, not above 0·5 units; urobilinogen

Blood.

Series No.	Type of case.	New lævulose method. Actual lævulose. (Mg. per 100 c.c.)		Ordinary lævulose method. Total reduction (MacLean). (Mg. per 100 c.c.)	Van den Bergh reaction. (In units per 100 c.c.)	Bilirubin content. (In units per 100 c.c.)	Bile-salts (plus 5 mg. adsorption per 100 c.c.) (Mg. per 100 c.c.)	Notes on blood examined. Blood-count, etc.
7	Acute alcoholic intoxication of liver	BL.	0	109	A prompt biphasic reaction	6·0	<5·0	..
		½ hr.	12	135				
		1 „	12	143				
		1½ hrs.	11	141				
		2 „	11	139				
8	Alcoholic cirrhosis	BL.	0	49	A weak delayed direct reaction	4·0	8·0	R.B.C. 4,200,000 W.B.C. 11,200 Hb 82% C.I. 0·90
		1 hr.	14	112				
		2 hrs.	10	76				
9	Old alcoholic (enlarged liver)	BL.	0	81	Slight indirect reaction	1·0	<5·0	..
		1 hr.	9	83				
		2 hrs.	5	82				
10	Acholuric jaundice	BL.	0	70	Very strong indirect reaction	50·0	6·0	R.B.C. 4,100,000 W.B.C. 8300 Hb 83% C.I. 1·0
		1 hr.	12	71				
		2 hrs.	7	65				
11	Catarrhal jaundice (partial obstruction)	BL.	0	96	A prompt biphasic reaction	11·0	10·0	..
		1 hr.	14	120				
		2 hrs.	10	99				
12	Catarrhal jaundice	BL.	0	60	Strong immediate direct reaction	30·0	12·0	..
		1 hr.	15	89				
		2 hrs.	15	78				
13	Cholecystitis	BL.	0	101	Mild immediate direct reaction	25·0	10·0	..
		1 hr.	12	121				
		2 hrs.	10	111				
14	Carcinoma of gall-bladder	BL.	0	111	Strong immediate direct reaction	60·0	21·0	..
		1 hr.	15	145				
		2 hrs.	13	120				
15	Carcinoma of head of pancreas	BL.	0	72	Very strong immediate direct reaction	70·0	23·0	..
		1 hr.	16	116				
		2 hrs.	14	110				
16	Intermittent jaundice (toxic)	BL.	0	..	Mild biphasic reaction	1·4	<5·0	..
		1 hr.	13	..				
		2 hrs.	9	..				
17	Subacute yellow atrophy	BL.	0	96	A biphasic reaction	4·0	6·0	..
		1 hr.	15	125				
		2 hrs.	10	112				
18	Hepatomegaly (20 g. lævulose given). Patient 5 years old	BL.	0	78	Indirect reaction	0·5	<5·0	R.B.C. 5,410,000 W.B.C. 20,400 Hb 98% C.I. 0·9
		1 hr.	7	112				
		2 hrs.	3	89				
19	Hypertrophic cirrhosis	BL.	0	99	An immediate direct reaction	10·0	9·0	R.B.C. 4,150,000 W.B.C. 4900 Hb 80% C.I. 0·90
		1 hr.	17	149				
		2 hrs.	16	133				
20	N.A.B. dermatitis and jaundice (past history of yellow fever)	BL.	0	96	A strong immediate direct reaction	60·0	15·0	R.B.C. 4,170,000 W.B.C. 3500 Hb 74% C.I. 0·89 Wassermann weakly positive.
		1 hr.	18	110				
		2 hrs.	15	104				
21	Pregnancy and vomiting (toxic jaundice)	BL.	0	125	A prompt delayed direct reaction	10·0	10·0	..
		1 hr.	17	151				
		2 hrs.	14	151				

Scott (1933); bile-salts, Scott (1934); bile-pigments, method shortly to be published from this laboratory. in fæces, 300 to 700 units. Bile-salts in blood, <5 mg. per 100 c.c.

	Urine.				Fæces.	
Notes on urine examined.	Bilirubin. (In units per 100 c.c.)	Bile-salts, Type A. (Mg. per 100 c.c.)	Bile-salts, Type B. (Mg. per 100 c.c.)	Urobilinogen. (In units per 100 c.c.)	Urobilinogen. (In units per 100 g. dry fæces.)	Differential fat analysis. (In g. per cent.)
Uroerythrin, large amount. Hay's test very faintly positive	0	0	0	5·0	5840	T.F. 10·9 S.F. 9·7 N.F. 1·2
Uroerythrin, small amount. Hay's test very faintly positive	0	0	0	9	3900	T.F. 9·3 S.F. 8·9 N.F. 0·4
Colour normal	0	0	0	1·7	Not obtainable.	
Uroerythrin, *nil.* Hay's test negative	0	0	0	10	6400	T.F. 24·2 S.F. 22·8 N.F. 1·4
Dark-coloured urine. Uroerythrin, *nil.* Hay's test moderately positive	2	1	0	2	200	T.F. 37·9 S.F. 36·1 N.F. 1·8
Uroerythrin fair amount. Hay's test faintly positive	4	5	0	0	0	T.F. 39·3 S.F. 8·6 N.F. 30·7
Uroerythrin, small amount. Hay's test, marked positive	2	2	0	9	1000	T.F. 23·4 S.F. 22·1 N.F. 1·3
Uroerythrin, faint trace. Hay's test moderately positive	15	4	0	0	0	T.F. 70·6 S.F. 68·3 N.F. 2·3
Dark-coloured urine. Hay's test faintly positive. Uroerythrin, faint trace. Diastase 100 units	30	5	0	0	0	T.F. 75·1 S.F. 68·1 N.F. 7·0
Uroerythrin, *nil.* Hay's test negative	0	0	0	2	450	T.F. .. S.F. .. N.F. ..
Uroerythrin, *nil.* Hay's test very faintly positive	0·2	0	1	3	2100	T.F. 22·6 S.F. 21·4 N.F. 1·2
Normal	0	0	0	1·2	710	T.F. 18·7 S.F. 17·1 N.F. 1·6
Very heavily pigmented urine. Uroerythrin, *nil.* Hay's test faintly positive	2	2	0	3	230	T.F. 32·3 S.F. 30·3 N.F. 2·0
Uroerythrin, fair amount. Hay's test very faintly positive	8	1	0	8	4500	T.F. 11·2 S.F. 9·5 N.F. ..
Uroerythrin, *nil.* Hay's test faintly positive. Three weeks pregnant by Aschheim-Zondek test	1·5	4	0	3	1840	T.F. 30·0 S.F. 23·0 N.F. 7·0

TABLE III.—*Lævulose Test in*

Blood.

Series No.	Type of case.	New lævulose method. Actual lævulose. (Mg. per 100 c.c.)		Ordinary lævulose method. Total reduction (MacLean). (Mg. per 100 c.c.)	Van den Bergh reaction. (In units per 100 c.c.)	Bilirubin content. (In units per 100 c.c.)	Bile-salts (plus 5 mg. adsorption per 100 c.c.) (Mg. per 100 c.c.)	Notes on blood examined. Blood-count, etc.
22	Pregnancy and nephritis	BL.	0	103	A biphasic reaction	8·0	11·0	Blood-urea, 48 mg. per 100 c.c.
		1 hr.	15	129				
		2 hrs.	13	121				
23	Secondary anæmia (sarcoma of liver)	BL.	0	107	Ditto	7·0	6·0	R.B.C. 2,470,000 W.B.C. 4900 Hb 36% C.I. 0·7 Platelets 138,000 (normal 200,000 to 400,000).
		1 hr.	14	133				
		2 hrs.	12	129				
24	Syphilitic gumma of liver	BL.	0	72	A strong immediate direct reaction	30·0	12·0	R.B.C. 4,950,000 W.B.C. 9900 Hb 92% C.I. 0·9 Wassermann +120
		1 hr.	17	101				
		2 hrs.	14	101				
25	Diabetes	BL.	0	256	Indirect reaction	0·4	50·0	..
		1 hr.	13	367				
		2 hrs.	8	317				
26	Subacute yellow atrophy and diabetes, 20.9.34	BL.	0	184	An immediate direct reaction	29·0	14·0	R.B.C. 2,260,000 W.B.C. 4900 Hb 40% C.I. 0·89
		1 hr.	14	232				
		2 hrs.	11	221				
26*a*	After the jaundice had entirely disappeared, 25.11.34	BL.	0	176	Indirect reaction	<0·5	<5·0	R.B.C. 4,400,000
		1 hr.	12	240				
		2 hrs.	7	217				

T.F. = Total fat; S.F. = split

TABLE V.—*Lævulose Test in*

Blood.

Series No.	Type of case.	New lævulose method. Actual lævulose. (Mg. per 100 c.c.)		Ordinary lævulose method. Total reduction (MacLean). (Mg. per 100 c.c.)	Van den Bergh reaction. (In units per 100 c.c.)	Bilirubin content. (In units per 100 c.c.)	Bile-salts (plus 5 mg. adsorption per 100 c.c.). (Mg. per 100 c.c.)	Notes on blood examined. Blood-count, etc.
27	Erythroderma (marked case)	BL.	0	97	Indirect reaction	0·6	6·0	R.B.C. 5,300,000 W.B.C. 9100 Hb 95% C.I. 0·90
		1 hr.	9	107				
		2 hrs.	4	100				
28	Prurigo nodularis	BL.	0	102	Ditto	0·3	<5·0	R.B.C. 5,200,000 W.B.C. 8700 Hb 84% C.I. 0·80
		1 hr.	12	126				
		2 hrs.	6	103				
29	Pernicious anæmia	BL.	0	91	„	15·0	7·0	R.B.C. 1,410,000 W.B.C. 3400 Hb 34% C.I. 1·2
		1 hr.	12	97				
		2 hrs.	6	93				
30	Ditto	BL.	0	99	„	19·0	8·0	R.B.C. 1,050,000 W.B.C. 3000 Hb 30% C.I. 1·4
		1 hr.	10	104				
		2 hrs.	4	100				
31	Hæmolytic anæmia) (origin obscure)	BL.	0	100	„	7·0	9·0	R.B.C. 2,030,000 W.B.C. 4800 Hb 40% C.I. 1·0
		1 hr.	9	129				
		2 hrs.	4	123				

Pathological Cases—continued.

Urine.					Fæces.	
Notes on urine examined.	Bilirubin. (In units per 100 c.c.)	Bile-salts, Type A. (Mg. per 100 c.c.)	Bile-salts, Type B. (Mg. per 100 c.c.)	Urobilinogen. (In units per 100 c.c.)	Urobilinogen. (In units per 100 g. dry fæces.)	Differential fat analysis. (In g. per cent.)
Uroerythrin *nil*. Hay's test very strongly positive. Albumen 0·34%	2	6	12	0	90	T.F. 36·3 S.F. 35·1 N.F. 1·2
Uroerythrin, large amount. Hay's test very faintly positive	0·5	3	0	6	2660	T.F. 18·7 S.F. 17·8 N.F. 0·9
Uroerythrin, *nil*. Hay's test very faintly positive	10	1	0	7	1500	T.F. 31·4 S.F. 28·0 N.F. 3·4
Pale-coloured urine	0	0	0	1·6	800	T.F. 30·3 S.F. 25·8 N.F. 4·5
Uroerythrin, *nil*. Hay's test moderately positive. Diastase 30 units	2	1	0	8	4400	T.F. 23·9 S.F. 12·0 N.F. 11·9
Normal	0	0	0	0·49	630	..

fat; N.F. = neutral fat.

Various Pathological Conditions.

Urine.					Fæces.	
Notes on urine examined.	Bilirubin. (In units per 100 c.c.)	Bile salts, Type A. (Mg. per 100 c.c.)	Bile salts, Type B. (Mg. per 100 c.c.)	Urobilinogen. (In units per 100 c.c.)	Urobilinogen. (In units per 100 g. dry fæces.)	Differential fat analysis. (In g. per cent.)
Uroerythrin, faint trace. Hay's test very faintly positive	0	0	2·5	1·2	890	T.F. 20·1 S.F. 19·4 N.F. 0·7
Colour normal	0	0	0	0	600	T.F. 16·7 S.F. 16·0 N.F. 0·7
Uroerythrin, large amount. Hay's test very faintly positive	0	0·5	0	7	3540	T.F. 23·4 S.F. 22·5 N.F. 0·9
Uroerythrin, *nil*. Hay's test faintly positive	0	2·2	0	9	4100	T.F. 16·4 S.F. 15·6 N.F. 0·8
Uroerythrin, *nil*. Hay's test very faintly positive	0	1·9	0	4	2600	T.F. 19·8 S.F. 19·1 N.F. 0·7

TABLE IV.—*Lævulose Test in a Case Complicated by Diabetes.*

Case: Subacute yellow atrophy and diabetes (see Table III, Cases 26 and 26*a*).	Venous.		Capillary.	
	Actual estimated lævulose. (Mg. per 100 c.c.)	Total reduction (Maclean's). (Mg. per 100 c.c.)	Actual estimated lævulose. (Mg. per 100 c.c.)	Total reduction (Maclean). (Mg. per 100 c.c.)
Before lævulose	0	184	0	186
1 hr. after	16	232	18	240
2 hrs. after	13	221	13	221
After two months, when the jaundice had entirely disappeared :				
Before lævulose	0	176	..	..
1 hr. after	12	240	..	..
2 hrs. after	7	217	..	..

clinically regarded as being possibly associated with some form of hepatic disorder. All these cases, however, gave normal values (Table V).

SUMMARY.

1. The lævulose tolerance test for liver function has been studied in relation to actual blood lævulose instead of the ordinary blood-sugar values.

2. This new approach simplifies greatly the interpretation of results, altogether forming the basis of a much more satisfactory means of determining deficiency in liver function.

3. In the presence of diabetes, which completely vitiates the usual form of tolerance test, only a slight effect is noted upon the lævulose figures and allowance for this may readily be made.

I wish to express my warmest thanks to Dr. J. Patterson for his interest and encouragement throughout the course of this investigation, and to Dr. R. A. Hickling, Physician to Charing Cross Hospital, in whose wards the liver cases were investigated.

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ERRATUM.

August number, p. 409, 3rd paragraph, delete lines 4 and 5.